บทบาทของระบบมัลติดรักซ์อิฟลักซ์ต่อการดื้อสารต้านจุลชีพใน *Acinetobacter baumannii* ที่แยกได้จากคนและสัตว์

นายสิรวิทย์ ภักดีพาณิชย์กิจ

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

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ROLE OF MULTIDRUG EFFLUX SYSTEMS IN ANTIMICROBIAL RESISTANCE IN *ACINETOBACTER BAUMANNII* CLINICAL ISOLATES FROM HUMANS AND ANIMALS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาบทบาทของระบบมัลติดรักซ์อิฟรักซ์ต่อการดื้อสารต้านจุลชีพใน *Acinetobacter baumannii* ที่แยกได้จากคนและสัตว์ โดยเชื้อ *Acinetobacter* spp. จำนวน 100 เชื้อจากคนนั้น ได้แก่เชื้อที่แยกได้จากผู้ป่วยที่เข้ารับการรักษาที่โรงพยาบาลศิริราชและโรงพยาบาลจุฬาลงกรณ์ และเชื้อ *A. baumannii* จำนวน 30 เชื้อจากสัตว์นั้นได้มาจากการเก็บตัวอย่างซากสัตว์จำนวน 210 ตัวอย่าง ซึ่งถูกส่งมาชันสูตรที่หน่วยพยาธิวิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย เชื้อจากคน (n=100) และเชื้อจากสัตว์ (n=30) ได้รับการตรวจยืนยันเป็น สปีชีส์ *baumannii* ด้วยวิธี Amplified Ribosomal DNA Restriction Analysis (ARDRA) i) เชื้อจากคนและสัตว์ทั้งหมดได้ถูกนำมาทำการทดสอบหาความไวรับต่อสารต้านจุลชีพที่ใช้ในการรักษาจำนวน 15 ชนิด รวมไปถึงศึกษาลักษณะการกระจายตัวของระบบมัลติดรักซ์อิฟรักซ์จากผลของ reserpine และ carbonyl cyanide *m*-chlorophenyl hydrozone (CCCP) และการแสดงออกของระบบมัลติดรักซ์อิฟรักซ์ พบว่าเชื้อส่วนใหญ่ทั้งที่แยกได้จากคน (98%) และจากสัตว์ (70%) เป็นเชื้อชนิดดื้อต่อยาปฏิชีวนะหลายกลุ่ม เชื้อที่แยกได้จากคน 53% และจากสัตว์ 6.7% ดื้อต่อยาทั้งหมดที่ใช้ในการศึกษานี้ CCCP มีผลในการเสริมประสิทธิภาพของสารต้านจุลชีพที่ดีกว่า reserpine รูปแบบการแสดงออกที่พบมากที่สุดคือ AdeB-AdeG-AdeJ และไม่พบการแสดงออกของยีน *adeE* ทั้งในเชื้อจากคนและสัตว์ ii) เชื้อจากคนและสัตว์จำนวน 25 เชื้อ (10 เชื้อจากคนและ 15 เชื้อจากสัตว์) ซึ่งครอบคลุมทุกรูปแบบการแสดงออกถูกเลือกมาเพื่อทำการศึกษาระดับการแสดงออกของระบบมัลติดรักซ์อิฟรักซ์ resistance-nodulation-cell division (RND) ด้วยวิธี quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) ร่วมกับดูลักษณะการกลายพันธุ์ของยีนควบคุมที่เกี่ยวข้องด้วยการวิเคราะห์การเปลี่ยนแปลงของนิวคลีโอไทด์ในโมเลกุลของดีเอ็นเอ ไม่พบการแสดงออกที่มากเกินในยีน *adeB* ในเชื้อทั้งจากคนและสัตว์ เชื้อจากคนและสัตว์จำนวน 2 เชื้อและ 10 เชื้อตามลำดับพบการแสดงออกที่มากเกินของยีน *adeG* แต่ไม่พบการกลายพันธุ์ใดๆของยีนควบคุม AdeL ในเชื้อดังกล่าว ตรวจพบระดับการแสดงออกของยีน *adeJ* เพิ่มขึ้น 0.4 ถึง 5.0 เท่าและ 0.1 ถึง 16.7 เท่าในเชื้อจากคนและสัตว์ตามลำดับ จากผลการศึกษาที่ได้ข้างต้นคาดว่าน่าจะมีกลไกการควบคุมการแสดงออกของ AdeABC, AdeFGH และ AdeIJK นอกเหนือไปจากยีนควบคุมที่ได้ทำการศึกษาในครั้งนี้ iii) เชื้อที่มีค่าความไวรับต่อสารต้านจุลชีพส่วนใหญ่ต่ำ (จากคน 10 เชื้อและจากสัตว์ 15 เชื้อ) ถูกเลือกเพื่อนำมาศึกษาบทบาทของระบบมัลติดรักซ์อิฟรักซ์ต่อการดื้อข้ามระหว่างสารฆ่าเชื้อและยาปฏิชีวนะโดยการให้เชื้อผ่านการรับสัมผัสกับ benzalkonium chloride, chlorhexidine and triclosan พบว่ามีเชื้อกลายพันธุ์ที่ดื้อต่อ triclosan ในเชื้อจากคนและจากสัตว์จำนวน 5 เชื้อและ 12 เชื้อตามลำดับ โดยเชื้อกลายพันธุ์ดังกล่าวมีการดื้อต่อยาปฏิชีวนะหลายชนิดเพิ่มขึ้น ระดับการแสดงออกของระบบมัลติดรักซ์อิฟรักซ์ไม่มีความสัมพันธ์กับระดับค่าความเข้มข้นต่ำสุดของยาที่สามารถยับยั้งการเจริญเติบโตของเชื้อ การกลายพันธุ์ที่พบในยีนควบคุม AdeL และ AdeN ไม่สอดคล้องกับการแสดงออกของมัลติดรักซ์อิฟรักซ์ ซึ่งน่าจะเป็นผลเนื่องมาจากระบบมัลติดรักซ์อิฟรักซ์ประเภท RND ไม่ได้เป็นกลไกหลักที่ทำให้เกิดการพัฒนาการดื้อต่อ triclosan ภายหลังในเชื้อ *A. baumannii* นี้ ผลที่ได้จากการศึกษาครั้งนี้สามารถสรุปได้ว่าเชื้อ MDR *A. baumannii* ที่มีการแสดงออกของระบบมัลติดรักซ์ อิฟรักซ์ประเภท RND มีการกระจายตัวและตรวจพบได้ทั่วไปทั้งในเชื้อจากคนและสัตว์ การรับสัมผัสต่อ triclosan สามารถทำให้เกิดการดื้อข้ามไปยังยาปฏิชีวนะซึ่งนำไปสู่การดื้อต่อยาปฏิชีวนะหลายกลุ่มในเชื้อ *A. baumannii* ได้ การศึกษาครั้งนี้แสดงให้เห็นว่าการใช้สารต้านจุลชีพและยาฆ่าเชื้ออย่างเหมาะสมควรได้รับการส่งเสริมให้เห็นถึงความสำคัญทั้งในทางการแพทย์และสัตวแพทย์

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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SIRAWIT PAGDEPANICHKIT: ROLE OF MULTIDRUG EFFLUX SYSTEMS IN ANTIMICROBIAL RESISTANCE IN *ACINETOBACTER BAUMANNII* CLINICAL ISOLATES FROM HUMANS AND ANIMALS. ADVISOR: ASSOC. PROF. RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D., 128 pp.

In this study, we aimed to determine the role of multidrug efflux systems in antimicrobial resistance in *Acinetobacter baumannii* isolates from humans and animals. For the human isolates 100 *Acinetobacter* spp. were obtained from patients admitted at Siriraj hospital and King Chulalongkorn Memorial hospital. For the animal isolates total of 210 animal samples were collected from animal carcasses submitted for necropsy at Pathology unit, Faculty of Veterinary Science, Chulalongkorn University and 30 *A. baumannii* were obtained. All human (n=100) and animal (n=30) isolates were confirmed as the *baumannii* species by using Amplified Ribosomal DNA Restriction Analysis (ARDRA). i) All the isolates were tested for antimicrobial susceptibilities to 15 clinically important antimicrobials, the contribution of multidrug efflux pump in the presence and absence of reserpine and carbonyl cyanide *m*-chlorophenylhydrozone (CCCP) and expression of multidrug efflux pumps. Most human (98%) and animal (70%) isolates were MDR. Fifty three percent of the human isolates and 6.7% of the animal isolates were resistant to all drugs tested. Effect of CCCP was more potent than reserpine enhancing of antimicrobial activity and regaining antimicrobial susceptibility. AdeB-AdeG-AdeJ was the predominant expression pattern and no *adeE* expression was observed in neither human nor animal isolates. ii) Twenty-five clinical isolates (10 human and 15 animal isolates), which cover all expression pattern were selected for determine transcription level of resistance-nodulation-cell division (RND) efflux systems by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and characterized for the regulatory mutations by DNA sequencing analysis. No overexpression of *adeB* was found in any human and animal isolates. Overexpression of *adeG* was observed in 2 and 10 of human and animal isolates, respectively. No mutation was identified in AdeL of all isolates overexpressing *adeG*. Expression level of *adeJ* was 0.4 to 5.0 fold and 0.1 to 16.7 fold in human and animal isolates, respectively. Therefore, the existence of additional regulatory mechanisms on AdeABC, AdeFGH and AdeIJK expression other that were not characterized in this study is suggested. iii) The isolates with low MIC values of most antimicrobial agents tested (10 human and 15 animal isolates) were selected for characterization of the contribution of multidrug efflux systems in biocide-antibiotic cross-resistance by step-wise passage to benzalkonium chloride, chlorhexidine and triclosan. Five and 12 triclosan-resistant mutants were obtained from the human and animal isolates, respectively. These triclosan-resistant mutant derivatives were additional resistant to several antibiotics. Expression level of efflux pumps was detected and was not corresponding to MIC level. The presence of mutation in AdeL and AdeN did not correlate to the expression of the corresponding efflux pumps. This suggested that transcription of RND efflux pumps could not be the sole mechanism for acquired resistance of triclosan in *A. baumannii* clinical isolates. In conclusion, the result showed that there is the wide spread of MDR *A. baumannii* and the wide distribution of RND efflux systems among the human and animal clinical isolates. Exposure to triclosan can promote cross-resistance to antibiotics, leading to multidrug resistance in *A. baumannii*. The data affirmed that the appropriate use of antimicrobials and biocides in human and veterinary medicine should be advocated.

Department: Veterinary Public Health Student's Signature .....
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LIST OF ABBREVIATIONS

A.	*Acinetobacter*
ABC	ATP-binding cassette superfamily
Acb	*A. calcoaceticus-baumannii*
AMK	amikacin
ARDRA	Amplified ribosomal DNA restriction analysis
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATM	aztreonam
bp	base pair(s)
BZK	benzalkonium chloride
ºC	degree(s) Celcius
CAR	carbenicillin
CAZ	ceftazidime
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
cDNA	complementary deoxyribonucleic acid(s)
CFU	colony-forming unit
CHG	chlorhexidine gluconate
CHL	chloramphenicol
CIP	ciprofloxacin
CLSI	the Clinical and Laboratory Standards Institute
Ct	threshold cycle
DNA	deoxyribonucleic acid(s)
DTT	dithiothreitol
dNTP	deoxyribonucleoside triphosphate(s)

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EDTA	Ethylenediaminetetraacetric acid
e.g.	*exempli gratia*
EPI	efflux pump inhibitor(s)
ERY	erythromycin
et al.	*et alibi* and others
GEN	gentamicin
h	hour(s)
ICU	intensive care unit
IDSA	the Infectious Diseases Society of American
i.e.	*id est*
IMP	inner membrane protein
KAN	kanamycin
LB	Luria-Bertani
MATE	multidrug and toxic compound extrusion
MDR	multidrug resistance
MFP	membrane fusion protein
MFS	major facilitator superfamily
MHA	Mueller-Hinton agar
MIC	Minimal Inhibitory Concentration
min	minute(s)
ml	milliliter(s)
mM	millimolar(s)
NARST	the National Antimicrobial Resistance Surveillance Thailand program
NEO	neomycin
ng	nanogram(s)
nm	nanometer(s)
OMP	outer membrane protein

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PCR	polymerase chain reaction
PIP	piperacillin
PMF	Proton Motive Force
rep-PCR	repetitive element palindromic polymerase chain reaction
RNA	ribonucleic acid(s)
RND	resistance-nodulation-cell division
rpm	rounds per minute
rRNA	ribosomal ribonucleic acid(s)
RT-PCR	reverse transcription polymerase chain reaction
s	second(s)
SMR	small multidrug resistance
SPE	spectinomycin
spp.	species
STR	streptomycin
TAE	Tris-acetate-EDTA
TCS	triclosan
TET	tetracycline
Tm	melting temperature
TMP	trimethoprim
TSAR	the Taiwan Surveillance of Antimicrobial Resistance program
U	unit
µg	microgram(s)
µl	microliter(s)
VAP	ventilator-associated pneumonia
v/v	volume per volume
w/v	weight per volume
ZOBA	Center of Zoonoses, Bacterial Animal Diseases and Antibiotic Resistance

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CHAPTER I

INTRODUCTION

1.1 Importance and Rationale

Acinetobacter baumannii is one of the most important opportunistic bacteria, which commonly cause outbreaks of nosocomial infection in hospitals (Peleg et al., 2008). The pathogen has been recognized as one of the top six deadliest microorganisms in the US by the Infectious Diseases Society of America (IDSA) (Sun et al., 2013). *A. baumannii* infection is common in patients in intensive care unit (ICU) and frequently involved in pneumonia, septicemia, meningitis and infections of the urinary tract, soft tissue and skin (McConnell et al., 2013). At the time of discovery in 1970's, *A. baumannii* was susceptible to most antimicrobial agents (Peleg et al., 2008). In contrast, most strains now become resistant to many antimicrobials commonly used in hospitals and infamously known as multidrug-resistant (MDR) *A. baumannii* (defined as being resistant at least 6 antimicrobial drugs (Gu et al., 2007; Poonsuk et al., 2012). Such resistance to a wide variety of antimicrobial agents causes difficulty in treatment, prolonged hospitalization and increased medical cost. As a result, therapeutic options for treatment of *A. baumannii* infection are very limited and *A. baumannii* infection usually ends up with treatment failure and fatal outcome (Sunenshine et al., 2007; Maragakis and Perl, 2008). Among the limited therapeutic choices, carbapenems and

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colistin have been used as the last resort for treatment of MDR *A. baumannii* (Gordon and Wareham, 2010). Unfortunately, carbapenem-resistant and colistin-resistant *A. baumannii* have emerged worldwide (Cai et al., 2012; Evans et al., 2013). This raises a particular concern that these will be no antibiotic treatment available for *A. baumannii* infection in the near future.

A. baumannii is known as not only a major problem of community-acquired infection in humans but also a causative agent for nosocomial infection in animals (Herivaux et al., 2016). Over the last few decades, the prevalence of MDR *A. baumannii* in humans has been increasing in both developing and developed countries worldwide. (Falagas et al., 2015) and is most commonly associated with hospital-acquired pneumonia (McConnell et al., 2013). A retrospective study in Italy showed that the prevalence of MDR *A. baumannii* was up to 54% among hospitalized patients and most were resistant to multiple classes of antimicrobial agents including ceftazidime, ciprofloxacin, imipenem and piperacillin/tazobactam (De Francesco et al., 2013). In Africa, the prevalence of MDR *A. baumannii* infection in patients admitted in hospitals in Algeria and Sudan was high up to 93.6% and 97%, respectively (Khorsi et al., 2015; Omer et al., 2015). A few studies from Taiwan reported very high infection rate (89%) of MDR *A. baumannii* in patients from 4 regional hospitals (Lin et al., 2013). Recently, MDR *A. baumannii* has been reported as the major gram-negative bacterium responsible for nosocomial infection in the Southeast Asia including Indonesia, Malaysia, Singapore, Thailand and Vietnam (Tjoa et al., 2013; Janahiraman et al., 2015;

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Suwantarat and Carroll, 2016). In Thailand, the National Antimicrobial Resistance Surveillance Thailand (NARST) program revealed that the prevalence of MDR *A. baumannii* has increased from 2.1% in 2000 to 46.7% in 2005 (Dejsirilert et al., 2009). MDR *A. baumannii* was the most common pathogen isolated from hospitals in Bangkok area of which its prevalence was 88.7-92.3% (Werarak et al., 2010; Chaisathaphol and Chayakulkeeree, 2014). The similar occurrence rate was observed among the university-based hospital in the northern region of Thailand (Chittawatanarat et al., 2014).

By 2000, *A. baumannii* infection in animals was scarcely reported (Peleg et al., 2008). *A. baumannii* was first described as a nosocomial pathogen for dogs and cats in an intensive care unit (Francey et al., 2000). Most of them were resistant to gentamicin, kanamycin, tetracycline, and trimethoprim-sulfonamide and responsible for 100% mortality rate in dogs with systemic infection (Francey et al., 2000). As indicated in human medicine, *A. baumannii* is also an important opportunistic pathogen in animals (Eveillard et al., 2013). MDR *A. baumannii* has been increasingly reported in dogs, cats, horses, cow and pigs in Europe and the United States (Vaneechoutte et al., 2000; Boerlin et al., 2001; Brachelente et al., 2007; Black et al., 2009; Zordan et al., 2011; Poirel et al., 2012; Zhang et al., 2013; Pomba et al., 2014; Herivaux et al., 2016). A study conducted in Switzerland demonstrated that *A. baumannii* animal isolates (dogs, cats and horses) from Center of Zoonoses, Bacterial Animal Diseases and Antibiotic Resistance (ZOBA) exhibited high resistance rate to ciprofloxacin (63.16%), gentamicin

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(89.47%), imipenem (10.53%) and tobramycin (15.79%) (Endimiani et al., 2011). Interestingly, the isolates were in the same clonal lineage with *A. baumannii* causing outbreaks in human in the same country (Endimiani et al., 2011). *A. baumannii* was reported not only in terrestrial animals, but also in diseased catfish in China (Xia et al., 2008), aquaculture environment of fish and shrimp farm in Southeast Asia (Huys et al., 2007) and environmental samples (including manured agricultural soils, pig slurry and sludge) in the UK and Reunion island (Byrne-Bailey et al., 2009; Pailhories et al., 2015). It was suggested that the circulation of *A. baumannii* between humans, animals and environment could be responsible for the emergence of community-acquired *A. baumannii* infection (Eveillard et al., 2013). It was also demonstrated that *A. baumannii* can be transmitted from animals to humans, and vice versa (Zordan et al., 2011; Belmonte et al., 2014). Therefore, animals should be monitored as an important reservoir of MDR *A. baumannii* (Endimiani et al., 2011).

A. baumannii exhibits resistance to antimicrobials via multiple mechanisms, of which active efflux system is one of the most common resistance machineries (Coyne et al., 2011). The multidrug efflux systems are capable of exporting board spectrum of antibiotics as well as some biocides (Webber and Piddock, 2003). Expression of an active efflux mediates resistance to several antimicrobial agents simultaneously, so called multidrug efflux pumps (Kumar and Schweizer, 2005). To date, there are 5 families of multidrug efflux systems that have been described in bacteria, including ATP-binding cassette (ABC) superfamily, Multidrug and toxic compound extrusion

(MATE) family, Major facilitator (MFS) superfamily, Small multidrug resistance (SMR) family and resistance-nodulation-cell division (RND) family (Sun et al., 2014). The RND efflux systems are most commonly found in Gram-negative bacteria, including *A. baumannii* (Paulsen et al., 1996; Poole, 2001). Up to date, 4 RND efflux systems have been identified in *A. baumannii* including AdeABC, AdeFGH, AdeIJK and AdeDE (Coyne et al., 2011; Hou et al., 2012). The AdeIJK is constitutively expressed and responsible for intrinsic resistance in *A. baumannii*, while the others are normally silent and overexpressed in regulatory mutants (Marchand et al., 2004; Damier-Piolle et al., 2008; Coyne et al., 2010b).

In addition to antibiotics, biocides e.g. triclosan, chlorhexidine, benzalkonium chloride are widely used in human and animal hospitals to control and remove microorganisms (Russell, 2002). Particular concern is that exposure to a biocide could cause resistance to that particular biocide in bacteria and promote cross-resistance to antibiotics (Russell et al., 1999). Cross-resistance between biocides and antibiotics has been demonstrated in many clinically-important bacteria e.g. *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* (Poole, 2002). It is well documented that multidrug efflux pumps are major mechanisms mediating biocide-antibiotic cross-resistance (Adair et al., 1971; Chuanchuen et al., 2001). Inactivation of AdeB and/or AdeJ lead to increased susceptibility of *A. baumannii* to benzalkonium chloride and chlorhexidine (Rajamohan et al., 2010b). Expression of the MFS efflux system AmvA leads to decreased susceptibility to erythromycin and several biocides including

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benzalkonium chloride and chlorhexidine (Rajamohan et al., 2010a). AbeM, the MATE efflux system, was shown to contribute to increase MICs of ciprofloxacin, gentamicin and triclosan (Su et al., 2005). However, data on cross-resistance between biocide and antimicrobial resistance is still limited in *A. baumannii*.

Multidrug efflux systems play an important role in antimicrobial resistance in medical-important bacteria. Inhibition of multidrug efflux systems that brings the efficacy of antibiotics back could benefit both human and veterinary medicine. Understand of the contribution, function and regulation of multidrug efflux systems is required for development of novel efflux pump inhibitors and other alternate therapeutic drugs. Such basic data is also essential for development of therapeutic guideline, prevention and control strategy to maximize treatment efficacy and minimize antimicrobial resistance in the pathogen (Garcia-Quintanilla et al., 2013). Even though *A. baumannii* is known as a clinically-important pathogen for a long time, little is known about multidrug efflux systems particularly in the clinical isolates. Therefore, this study aimed to investigate role of multidrug efflux systems in multiple resistance and their contribution to cross-resistance in *A. baumannii* clinical isolates from humans and animals.

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1.2 Research Objectives

1.2.1 To determine the contribution of the proton-dependent efflux pumps systems in *A. baumannii* clinical isolates from humans and animals

1.2.2 To examine distribution, expression and regulation of the RND multidrug efflux systems in MDR *A. baumannii* clinical isolates from humans and animals

1.2.3 To characterize cross-resistance between biocides and antibiotics via multidrug efflux systems in *A. baumannii* clinical isolates from humans and animals


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1.3 Research Benefits

1.3.1 Novel knowledge

- Antimicrobial resistance profile of *A. baumannii* clinical isolates from humans and animals in Thailand.
- Genetic characteristics of antimicrobial resistance associated with multidrug efflux systems of *A. baumannii* clinical isolates from humans and animals in Thailand.

1.3.2 Application of knowledge

- Antimicrobial resistance data can be used as part of antimicrobial resistance surveillance.
- Both phenotypic and genotypic data can be applied for risk assessment when *A. baumannii* is used as a study model.
- Both data and the strains isolated in this study can be used for further experiments e.g. development of efflux pump inhibitor, diagnostic tool for *A. baumannii* infection and treatment.
- The data are used to support the development of guideline for prudent use of antibiotics in humans and animals.

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CHAPTER II

LITERATURE REVIEW

2.1 General characteristics of *A. baumannii*

Acinetobacter spp. is Gram-negative coccobacilli bacterium that is non-fermentative, strictly aerobe, non-motile and non-pigmented. It is catalase-positive and oxidase-negative (Peleg et al., 2008). This organism has been classified into the Pseudomonadales order and the Moraxellaceae family. The genus *Acinetobacter* contains at least 33 genomic species with some unnamed or ungrouped strains (O'Shea, 2012). Among these species, *A. baumannii* is the most clinically important species and needs to be identified for medical advantages. However, identification of *Acinetobacter* species by using phenotype-based techniques generates unreliable results and cannot differentiate the *A. calcoaceticus-baumannii* complex (*Acb* complex). Amplified ribosomal DNA restriction analysis (ARDRA) is a reference methods validated for differentiation of *A. baumannii* from other *Acinetobacter* spp. (Vaneechoutte et al., 1995; Dijkshoorn et al., 1998). *A. baumannii* widely distributes in environment such as soil and water (Houang et al., 2001). Moreover, it was found on human skin (Seifert et al., 1997) and in human feces (Dijkshoorn et al., 2005). In addition, *A. baumannii* has been isolated from dogs and cats in intensive care units (ICU) (Francey et al., 2000).

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2.2 Pathogenesis of *A. baumannii*

A. baumannii has emerged as one of the most important opportunistic pathogens causing nosocomial infections in humans and animals. Recently, the presence of community- and hospital-acquired *A. baumannii* infection was increasing in humans. Similarly, life-threatening hospital-acquired infection in animals has been reported (Francey et al., 2000). Several factors contribute to its ability to become a successful pathogen, including survival and growth on solid and dry surfaces, adhesion to biotic and abiotic materials and biofilm formation in colonization process (Gaddy and Actis, 2009). Furthermore, the ability to obtain iron for nutrients and attach to and destroy epithelial cells by production of enzyme gelatinase in some strains results in damaged host tissues (Cevahir et al., 2008).

In humans, infection with *A. baumannii* is commonly found in patients admitted in ICU, including patients with ventilator-associated pneumonia (VAP), urinary-tract infections, skin, soft tissue and wound infections and immunocompromised conditions (Peleg et al., 2008). This pathogen was identified in injured soldiers, who went operated in Iraq and Afghanistan with gunshot wounds, explosive wounds and burn wounds (Murray, 2008). It was also isolated from wounded survivors of natural disasters such as tsunami in Southeast Asia (Garzoni et al., 2005).

A. baumannii infection in animals has been reported in pets such as dogs, cats and horses with infection in various organs (e.g. respiratory tract, urinary system, wound and blood stream) (Francey et al., 2000; Vaneechoutte et al., 2000). In addition, this

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organism was found as a co-infection pathogen in cutaneous mycobacteriosis lesion in falcons (Muller et al., 2010).

2.3 Antimicrobial resistance in *A. baumannii*

A. baumannii cases in humans are mostly involved in ICU patients or hospital-acquired pneumonia patients (Peleg et al., 2008). Multidrug-resistant (MDR) *A. baumannii* that is defined as *A. baumannii* resistant to at least six or more antimicrobial agents have increasingly emerged worldwide, including in USA, Europe, China and South Pacific (Perez et al., 2007; Magiorakos et al., 2012). *A. baumannii* develops resistance to several classes of antimicrobial agents via multiple mechanisms (Nordmann and Poirel, 2009). Importantly, it is naturally resistant to the commonly-used antimicrobials by its intrinsic resistance property.

A previous study showed that *A. baumannii* isolates collected from hospitals in 14 European countries exhibited imipenem-resistance rate up to 48.9% (Nordmann et al., 2011). In Asia, spread of MDR *A. baumannii* has been reported in several countries (Chang et al., 2012; Xu et al., 2013). The Taiwan Surveillance of Antimicrobial Resistance (TSAR) V program demonstrated high resistance rates of *A. baumannii* to ciprofloxacin, amikacin and imipenem. These resistance rates were significantly higher than those previously reported (Chuang et al., 2013). The National Antimicrobial Resistance Surveillance Thailand (NARST) program reported prevalence of MDR *A.baumannii* in Thailand increased from 2.1% in 2000 to 46.7% in 2005 (Dejsirilert et

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al., 2009). The prospective study of nosocomial pneumonia caused by *A. baumannii* revealed that most of the clinical isolates from tertiary care hospitals were resistant to many antimicrobials simultaneously, including carbapenems (Werarak et al., 2012).

In comparison to the reports in humans, the research data of *A. baumannii* in animal is less. The very first report was in *Acinetobacter* spp. isolated from gingival flora of the dog and was insignificantly concerned (Saphir and Carter, 1976). Later, *A. baumannii* cases in animals have been increasingly reported. The horse isolates in an equine clinic were found to be resistant to many antibiotics including amoxicillin, amoxicillin-clavulanic acid, ceftiofur, tetracyclines and sulfonamides (Vaneechoutte et al., 2000). The recent report of *A. baumannii* isolates of veterinary origin in Switzerland showed resistance to ciprofloxacin, gentamicin, imipenem and tobramycin at the rate of 63.16%, 89.47%, 10.53% and 15.79%, respectively. It was suggested that animals should be monitored as an important reservoir of MDR *A. baumannii* (Endimiani et al., 2011).

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2.4 Genetics of antimicrobial resistance in *A. baumannii*

A. baumannii exhibits antimicrobial resistance via multiple mechanisms, mainly divided to intrinsic and acquired resistance. *A. baumannii* is infamous for its high-intrinsic resistance that is primarily due to the synergy of low membrane permeability and basal-level expression of efflux pumps (Vila et al., 2007). The important mechanisms leading to acquired multidrug resistance include acquisition of

transferable resistance elements (e.g. integrons containing resistant gene cassettes) and overexpression of chromosomally-encoded efflux systems, modification of target sites and drugs modification or inactivation (Coyne et al., 2011).

2.5 Multidrug efflux systems

Multidrug efflux system is one of the most common resistance mechanisms in Gram-negative bacteria including *A. baumannii* (Coyne et al., 2011). Efflux-mediated resistance could occur in both intrinsic and acquired manner. Bacterial efflux systems are classified into 5 superfamilies; the major facilitator (MFS) superfamily, the ATP-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family and the resistance-nodulation-cell division (RND) family. Among these efflux families, the RND family efflux systems are most commonly found in Gram-negative bacteria (Poole, 2001). All members of the RND efflux family export the target agents by a substrate-proton (H^+) antiport mechanism and are capable of extruding board spectrum of antimicrobial agents (Kumar and Schweizer, 2005). The RND efflux pumps generally operate as a tripartite system, composing of an inner membrane protein (IMP) that is multidrug transporter, a membrane fusion protein (MFP) that is periplasmic accessory protein, and an outer membrane protein (OMP) that is an exit for substrates (Eswaran et al., 2004). The RND efflux systems identified in *Acinetobacter* spp. are AdeABC, AdeFGH, AdeIJK and AdeDE as shown in figure 1 and 2. The first three systems are specifically found in *A. baumannii*

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but the last one is present in *Acinetobacter* spp. (Chu et al., 2006; Cortez-Cordova, 2010). Moreover, non-RND efflux systems including MFS and MATE families have also been demonstrated to be associated with antimicrobial efflux, which are AmvA and AbeM, respectively (Coyne et al., 2011).

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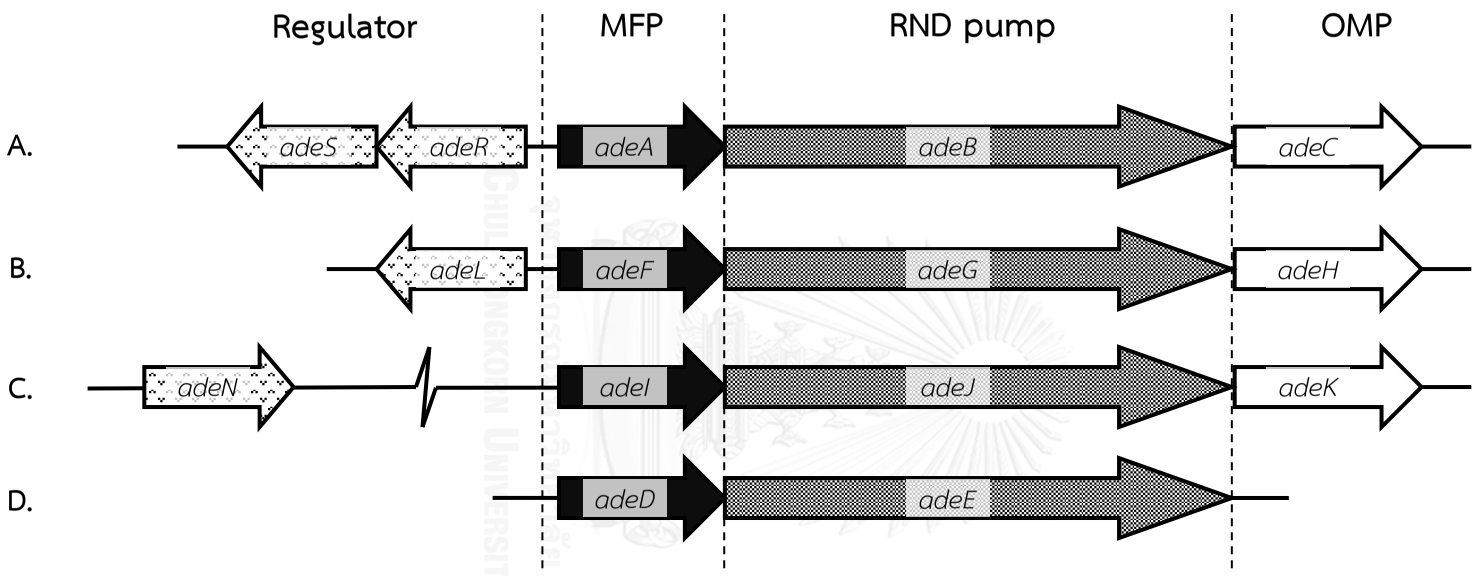

Figure 1: The operons of RND efflux systems in *A. baumannii*. A. The *adeABC* multidrug efflux pump and it regulator *adeRS* that are located upstream and transcribed in opposite direction; B. The *adeFGH* multidrug efflux pump and it regulator *adeL* that are located upstream and transcribed in opposite direction; C. The *adeIJK* multidrug efflux pump and it regulator *adeN* that are located 813 upstream from *adeI*; D. The *adeDE* multidrug efflux pump, of which genes encoding for outer membrane protein and regulatory protein are not located on the same operon.

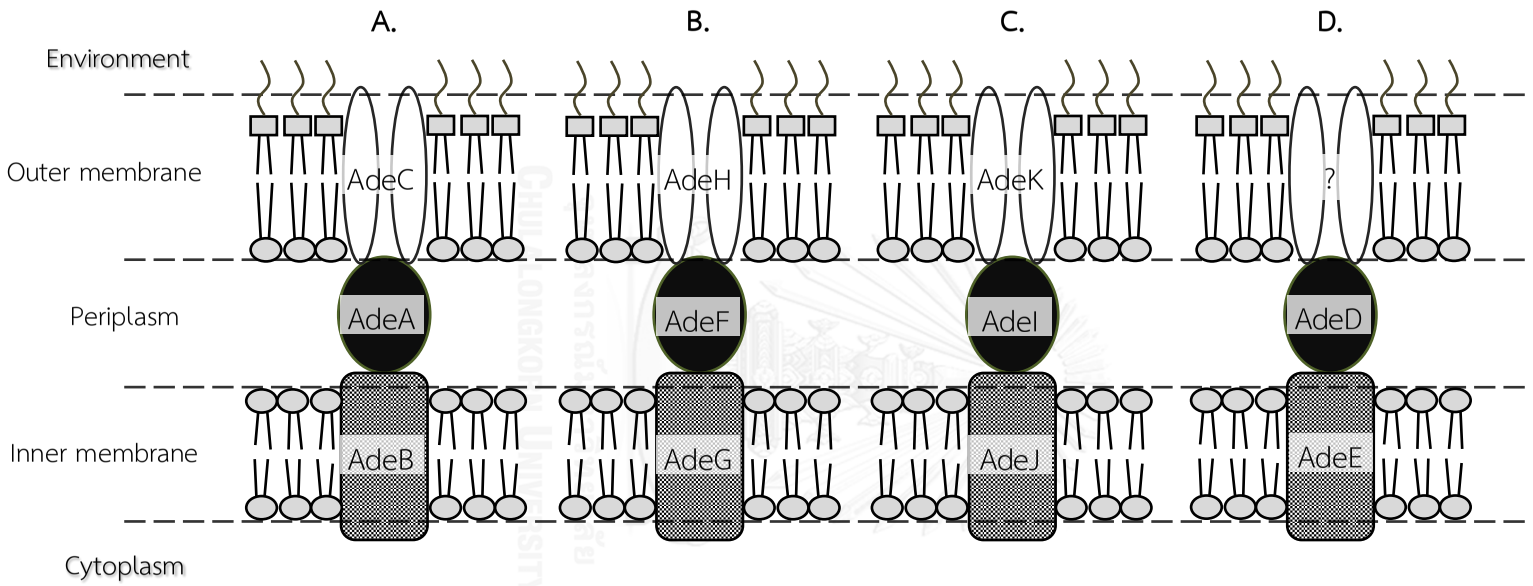

Figure 2: Structure of the tripartite-RND efflux systems. A. The AdeABC multidrug efflux system; B. The AdeFGH multidrug efflux system; C. The AdeIJK multidrug efflux system; D. The AdeDE multidrug efflux system. Membrane fusion protein encoded (MFP) by *adeA*, *adeF*, *adeI* and adeD. Inner membrane protein (IMP) encoded by *adeB*, *adeG*, *adeJ* and *adeE*. And outer membrane protein (OMP) encoded by *adeC*, *adeH* and *adeK* (modified from Poole, 2005).

2.5.1 The AdeABC multidrug efflux system

The first characterized RND efflux system in *A. baumannii* was AdeABC (Magnet et al., 2001). This efflux system is encoded by the *adeABC* operon, consisting of *adeA*, *adeB* and *adeC* gene encoding for a membrane fusion protein, AdeA; an inner membrane protein, AdeB; and an outer membrane protein, AdeC; The *adeABC* operon is located on the chromosome (Marchand et al., 2004). Overexpression of this efflux system may lead to MDR phenotype of *A. baumannii*. Inactivation of the operon in clinical isolates resulted in increased susceptibility to aminoglycosides, β-lactams, chloramphenicol, fluoroquinolones, macrolides, tetracyclines, tigecycline, and trimethoprim (Magnet et al., 2001; Damier-Piolle et al., 2008).

Expression of *adeABC* is regulated by two regulatory genes, *adeR* and *adeS*, the regulators in two-component of the operon systems that are located upstream and transcribed in the opposite direction (Marchand et al., 2004). Inactivation of or mutation in *adeR* or *adeS* leads to constitutive expression of AdeABC (Marchand et al., 2004). Mutations previously identified were Pro116-Leu in AdeR and Thr153-Met in AdeS, resulting in overexpression of *adeABC* (Marchand et al., 2004).

2.5.2 The AdeFGH multidrug efflux system

AdeFGH is encoded by the *adeFGH* operon. AdeF, AdeG and AdeH are membrane fusion protein, inner membrane protein and outer membrane protein, respectively (Coyne et al., 2010b). Overexpression of this efflux system results in high-

level resistance to chloramphenicol, clindamycin, fluoroquinolones and trimethoprim and decreased susceptibility to sulfamethoxazole, tetracycline and tigecycline (Coyne et al., 2010b).

The regulatory gene of AdeFGH is *adeL*, which is located upstream and transcribed in opposite direction of *adeFGH* (Coyne et al., 2010b). Mutation in *adeL* leads to overexpression of AdeFGH and mutations previously identified were Thr319-Lys and Val391-Gly (Coyne et al., 2010b).

2.5.3 The AdeIJK multidrug efflux system

The AdeIJK efflux system is encoded by the *adeIJK* operon, consisting of AdeI, a membrane fusion protein; AdeJ, an inner membrane protein and AdeK, an outer membrane protein (Damier-Piolle et al., 2008). This RND efflux system is responsible for intrinsic resistance and also involved in exporting of various antimicrobial agents e.g. ß-lactams, chloramphenicol, cotrimoxazole, fluoroquinolones, fusidic acid, lincosamides, novobiocin, rifampin, tetracyclines and tigecycline (Damier-Piolle et al., 2008). Base on a previously study, the expression level of AdeIJK was lower than that of AdeABC, and it is likely that overexpression of AdeIJK was self-limited due to the possible damages to the host cells (Coyne et al., 2010a).

The regulatory genes of *adeIJK* are not adjacent to the pump operon and regulation of this efflux system remains unclear (Coyne et al., 2010b). However, whole-genome sequencing revealed *adeN*, a regulatory protein in a TetR family, that

is located 813 upstream from *adeI* (Rosenfeld et al., 2012). Inactivation of *adeN* in susceptible strains resulted in 5-fold increased expression level of *adeJ*, and increased MICs of ß-lactams, chloramphenicol, erythromycin, quinolones, sulfonamides and tetracycline. This suggested role of *adeN* as a negative response of *adeJ* (Rosenfeld et al., 2012).

2.5.4 The AdeDE multidrug efflux system

The AdeDE efflux system is encoded by the *adeDE* operon. The *adeD* and *adeE* gene encode for membrane fusion protein and inner membrane protein, respectively. Outer membrane protein encoded gene is not clustered together with *adeD* and *adeE* and currently not identified (Chau et al., 2004). This efflux system may be responsible for export of various drugs including amikacin, ceftazidime, chloramphenicol, ciprofloxacin, erythromycin, meropenem, rifampin and tetracycline (Chau et al., 2004). In general, *adeDE* was mostly found in *Acinetobacter* genomic DNA group 3 (Chau et al., 2004; Chu et al., 2006). Some study reported the presence of the *adeDE* operon and its expression in *A. baumannii* clinical isolates (Cortez-Cordova, 2010; Hou et al., 2012).

2.5.5 The AmvA multidrug efflux system

The AmvA efflux pump is a member of the MFS efflux system. This MFS drug transporter is classified as drug:H^+ antiporter-2 (DHA2) family, with 14-transmembrane-domain system (Paulsen et al., 1996). Deletion inactivation of the

amvA gene resulted in increased susceptibility to benzalkonium chloride, chlorhexidine, ciprofloxacin, erythromycin, norfloxacin and novobiocin (Rajamohan et al., 2010a). In addition, the expression level of AmvA was found to be higher in antimicrobial resistant strains, which exhibited very high MICs of aminoglycosides, carbapenems, cephalosporins and fluoroquinolones (Rajamohan et al., 2010a).

2.5.6 The AbeM multidrug efflux system

AbeM is an H^+ -driven drug efflux pump, which is a member of the MATE family transporter (Su et al., 2005). This efflux pump extrudes triclosan, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, kanamycin, norfloxacin, ofloxacin and trimethoprim (Su et al., 2005). However, the expression of *abeM* was found in most of clinical isolates without any correlation with antimicrobial resistance (Bratu et al., 2008; Chen et al., 2009).

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2.6 Inhibition of multidrug efflux pumps by efflux pump inhibitor (EPI)

Since multidrug efflux systems are one of the most important mechanisms responsible for antimicrobial resistance in *A. baumannii*, inhibition of their function could benefit both animal and human medicine. Efflux pump inhibitors (EPIs) are present as a reasonable treatment option to fight against the pathogens (Wieczorek et al., 2008). Using of EPIs not only make ineffective drugs turn into effective use, but also reduce the emergence of MDR organisms (Stavri et al., 2007).

In the present, few EPIs have been studied include carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), reserpine and phenyl-arginine-ß-naphthylamide (PAßN). Addition of reserpine and CCCP with fluoroquinolones resulted in decreased their resistance rates and suggested both reserpine and CCCP can inhibit efflux systems of *A. baumannii* (Shi et al., 2005). However, the effects of reserpine and CCCP were not specific to RND efflux systems (Zechini and Versace, 2009). Both chemicals can impede all mechanisms that utilize proton-motive force as energy. PAßN exhibits specific inhibition activity to the RND efflux systems (Pannek et al., 2006). Exposure to PAßN resulted in decreased MICs of tigecycline, gentamicin, tobramycin, chloramphenicol and several ß-lactams (Peleg et al., 2007). In addition, PAßN has been shown to have the ability to inhibit the function of the AdeFGH efflux system in exporting few antimicrobial agents including chloramphenicol, clindamycin and trimethoprim (Cortez-Cordova and Kumar, 2011).

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2.7 Cross-resistance between biocides and antibiotics

Exhibit of antibiotic resistance can occur via various mechanisms, which provide resistance to specific or broad range of antibiotics (Poole, 2002). In general, biocides have been commonly used for disinfection and sterilization application. However, biocide-resistance organisms have been increasingly emerged (Poole, 2005). Cross-resistance between biocides and antibiotics has been reported in some clinically-important pathogens e.g. *Pseudomonas aeruginosa*, *Salmonella*, *Escherichia coli*

(Chuanchuen et al., 2001; Poole, 2002; Brenwald and Fraise, 2003). Evidence showed that the RND efflux systems play an important role in multidrug resistance of *A. baumannii* and mediate cross-resistance between antibiotics and biocides (Rajamohan et al., 2010b). Inactivation efflux system encoded gene could affect both antibiotic and biocide susceptibilities (Poole, 2005). Exposure to disinfectant could select for disinfectant-resistant isolates that also exhibit resistance to antibiotics (Russell et al., 1999). In *A. baumannii*, overexpression of AdeABC and AdeIJK was found to be involved in increased biocide MICs in clinical isolates (Rajamohan et al., 2010b). In addition, non-RND efflux systems i.e. AmvA and AbeM are responsible for extrude both biocides and antibiotics (Su et al., 2005; Rajamohan et al., 2010a). Concurrently, these efflux systems are capable of exporting antibiotics. Therefore, they are likely to mediate cross-resistance between antibiotics and biocides (Yavari et al., 2013).

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CHAPTER III

MATERIALS AND METHODS

The experiment was divided into 4 phases, including 3.1) Collection and isolation of *A. baumannii* clinical isolates from human and animal; 3.2) Determination of antimicrobial resistant profile and RND multidrug efflux systems expression pattern; 3.3) Characterization of expression and regulation of the RND multidrug efflux systems; and 3.4) Characterization of the contribution of the RND multidrug efflux systems in biocide-antibiotic cross-resistance as shown in figure 3.

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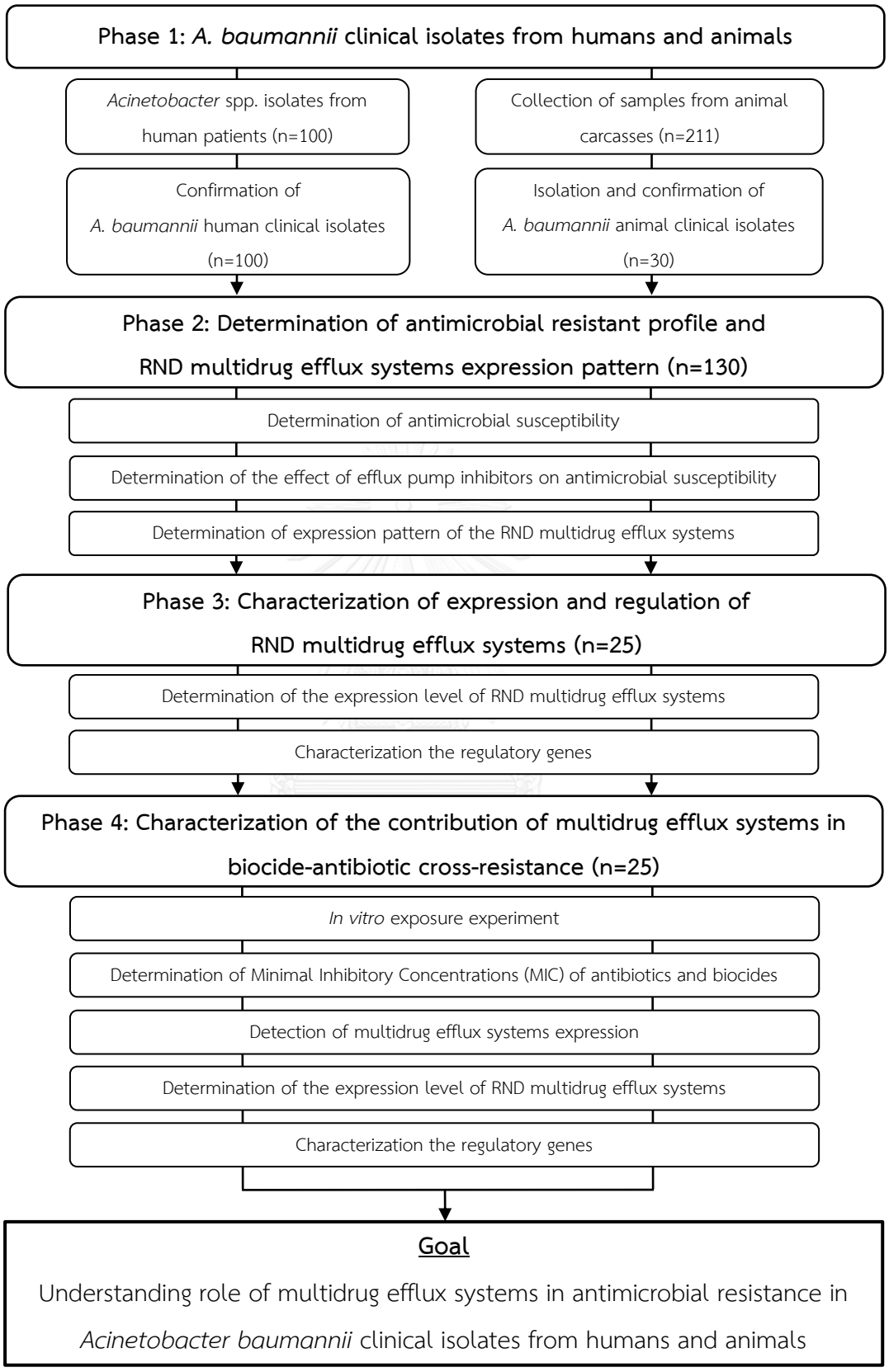

Figure 3: Experimental outline

3.1 Sample collection and isolation of *A. baumannii* clinical isolates from humans and animals

3.1.1 *A. baumannii* clinical isolates from humans

3.1.1.1 *Acinetobacter* spp. isolates from human patients

One hundred *Acinetobacter* spp. human isolates were obtained from the bacterial strain collection of Department of Microbiology, Faculty of Medicine Siriraj hospital, Mahidol University, Bangkok. The isolates were collected from various type of specimens, including sputum (n=56), skin wound (n=16), blood (n=8), urine (n=7), catheter tips (n=4), pus (n=3), penis (n=2), cerebrospinal fluid (CSF) (n=1), endotracheal tube (n=1), peritoneal fluid (n=1), pleural fluid (n=1). The specimens were obtained from patients admitted at Siriraj hospital and King Chulalongkorn Memorial hospital during December 2001 to May 2008. The human clinical isolates were identified as *Acinetobacter* spp. by using the VITEK GNI card (BioMérieux Vitek, MO, USA) and the API 20NE system (BioMérieux, MO, USA) at Department of Microbiology, Faculty of Medicine, Siriraj hospital, Mahidol University. The isolates were stored at -80°C in Luria-Bertani broth (LB, Difco™, NJ, USA) supplemented with 20% glycerol and transferred to Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University for further studies.

3.1.1.2 Confirmation of *A. baumannii* human clinical isolates

All of the human isolates were confirmed for species *baumannii* by using Amplified Ribosomal DNA Restriction Analysis (ARDRA) as previously described

(Vaneechoutte et al., 1995; Dijkshoorn et al., 1998). The isolates from -80ºC stock were grown on LB agar (Difco™, NJ, USA), incubate at 37ºC overnight. A loopful of bacterial colony was suspended into 50 µl of sterile distilled water. The suspension was boiled for 10 min and centrifuge at 12,000 rpm for 5 min. Forty µl of supernatant was used as chromosomal DNA template and stored at -20 ºC until used. The 16S rRNA gene was amplified using conventional PCR with specific primer pairs Universalprimerup and Universalprimerdown as shown in Table 1. A total 50 µl of PCR reaction consisted of 25 µl of 2x KAPA Taq ReadyMix (1 U KAPA Taq DNA Polymerase, 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP/reaction) (Kapa Biosystems, MA, USA), 2.5 µl of 10 µM Universalprimerup primer, 2.5 µl of 10 µM universalprimerdown, 18 µl of nuclease-free water and 2 µl of chromosomal DNA template. The PCR reaction was carried out at 95ºC for 5 min, followed by 35 cycles of 95ºC for 45 s, 50ºC for 45 s, 72ºC for 1 min and final extension at 72ºC for 7 min in Tpersonal combi model® (Biometra, Göttingen, Germany). Amplified PCR products were checked for yield and specific size (~1,500 bp) by agarose gel electrophoresis (1.5% w/v) stained with ethidium bromide. The amplified PCR products were used for restriction digestion with restriction enzymes, including *Alu*I, *Cfo*I and *Mbo*I (Thermo Fisher Scientific, MA, USA). The restriction digestion was performed using Tpersonal combi model® (Biometra, Göttingen, Germany) at 37ºC for 90 min in total 20 µl contained 2 µl of suitable commercial buffer for each enzyme, 10 µl of PCR product (0.1-0.5 µg of DNA), 7.5 µl of nuclease-free water and 0.5 µl of each enzyme (10 U/µl). The restriction digestion was stopped by

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heat inactivation at 65ºC for *Alu*I and *Mbo*I or 80ºC for *Cfo*I for 20 min. Restriction digestion patterns were analyzed by Owl™ EasyCast™ B2 Mini Gel Electrophoresis Systems (Thermo Fisher Scientific, MA, USA) on 2% w/v agarose gel with 1x Tris Acetate-EDTA (TAE) buffer. The agarose gel was visualized by ethidium bromide stained in Bio-Rad Gel Documentation system (Bio-Rad Laboratories, CA, USA). All of the *A. baumannii* human isolates were stored at -80ºC in LB broth (Difco™, NJ, USA) supplemented with 20% glycerol until used

3.1.2 *A. baumannii* clinical isolates from animals

3.1.2.1 Collection of samples from animal carcasses

The number of isolates were obtained from all 210 samples, which including dog (n=112), cat (n=45), rabbit (n=17), bird (n=17), pig (n=5), Hedgehog (n=4), lizard (n=3), monkey (n=2), hamster (n=3), gatsby (n=1), chinchilla (n=1). The animal samples were obtained from animal carcasses that submitted for necropsy at Pathology Unit, Faculty of Veterinary Science, Chulalongkorn University during September 2013 to September 2014. The whole animal carcasses were received and stored at 2-4ºC within 4 h after the animal's death. The sample collection was performed by nasal swab within 8 h after animal carcasses received and direct plated on CHROMagar™ Acinetobacter medium (CHROMagar, Paris, France) immediately, incubate at 37ºC for 18-24 h. Any dead animals with respiratory infections and chronic diseases such as heart disease, cancer and diabetes were sampled. Source of the animal carcasses were from different places including Small Animal Teaching Hospital, Faculty of Veterinary

Science, Chulalongkorn University (n=164), private animal hospital (n=21), bird farm (n=14), pig farm (n=5), Dusit zoo (n=2) and by owner (n=4).

3.1.2.2 Isolation and confirmation of *A. baumannii* from animal clinical isolates

Typical red colony on the incubated plates, which referred to *Acinetobacter* spp. was isolated by streaked on LB agar and incubated at 37ºC overnight. The *Acinetobacter* spp. animal isolates were confirmed as *A. baumannii* species by using ARDRA method as described above. All of the *A. baumannii* animal isolates were stored at -80ºC in LB broth supplemented with 20% glycerol until used.

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Table 1: Primers used in this study

Primer name	Sequence (5' to 3')	Gene	Tm (°C)	Reference
Universalprimerup	TGGCTCAGATTGAACGCTGGCGGC	*16S rRNA*	50	Vaneechoutte et al. (1995)
Universalprimerdown	TACCTTGTTACGACTTCACCCCA			
AdeB_Fw_P5	CCCTAATCAAGGACGTATGC	*adeB*	61	This study
AdeB_RV_P6	GGTGCCTTATTCCATTGTGG			
AdeG_Fw_P7	TTATCAGGTCCGTGCACAAG	*adeG*	61	This study
AdeG_RV_P8	GTGCAGCAATACGTTCAACC			
AdeJ_Fw_P9	GCCGTATGATGCCTGAAGAC	*adeJ*	62	This study
AdeJ_Rv_P10	GCAGCCAAGCAAAGGAATAC			
AdeE_Fw_P11	CAGGGACGTGTTAAGCGAGT	*adeE*	65	This study
AdeE_Rv_P12	CCAGACTTGCAGCCACTACA			
AdeR_Fw_P15	GTTAAGGCAATAAAAAGTTGCTT	*adeR*	52	This study
AdeR_Rv_P16	TGGAGTAAGTGTGGAGAAATACG			
AdeS_Fw_P17	CTTGGTTAGGTTAGATATGGCATT	*adeS*	54	This study
AdeS_Rv_P18	GGCGTGGGATATAGGCTAGATAA			
AdeL_Fw_P19	ATTTCGAACTTACTCATATGCTGA	*adeL*	53	This study
AdeL_Rv_P20	GGAATGGACGGAGCATAAAA			
AdeN_Fw_P21	AAGCAGTGTTAGCCGTCGTT	*adeN*	58	This study
AdeN_Rv_P22	GTTGTTGGCTGGGTGGAAGT			
AmvA_Fw_P23	AATCCTGACAATTATCGTCCTCA	*amvA*	60	This study
AmvA_Rv_P24	TCTTCGGAAAACTAAACCAGACA			
AbeM_Fw_P25	TGTCGAATGTCACGTCGTTT	*abeM*	59	This study
AbeM_Rv_P26	CGTTTGGTATTCAAATAGAGTCG			
rpsLrealtimeup	CGGCACTGCGTAAGGTATGC	*rpsL*	62	Chuanchuen et al. (2008)
rpsLrealtimedown	CCCGGAAGGTCCTTTACACG			
ERIC IR	ATGTAAGCTCCTGGGGATTCAC	Rep-PCR	45	Amonsin et al. (2003)
ERIC II	AAGTAAGTGACTGGGGTGAGCCG			

3.2 Determination of antimicrobial resistance profile and RND multidrug efflux systems expression pattern

3.2.1 Determination of antimicrobial susceptibility

Minimal Inhibitory Concentrations (MICs) were determined by using 2-fold agar dilution method according to Clinical and Laboratory Standard Institute guideline (CLSI, 2010). Fifteen clinically important antimicrobials were used in this study including amikacin (AMK), aztreonam (ATM), carbenicillin (CAR), ceftazidime (CAZ), chloramphenicol (CHL), ciprofloxacin (CIP), erythromycin (ERY), gentamicin (GEN), kanamycin (KAN), neomycin (NEO), piperacillin (PIP), spectinomycin (SPE), streptomycin (STR), tetracycline (TET) and trimethoprim (TMP). All of the antimicrobials were purchased from Sigma-Aldrich® (MO, USA). The MIC breakpoints according to CLSI (CLSI, 2010; CLSI, 2013) are used to define the isolates as resistant or susceptible. Antimicrobial agent stock solutions were prepared by appropriate solvents and diluents (Table 2). *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were used for quality control strains.

Agar dilution procedure as described by CLSI (2010) was used in this study. The isolates were streaked on Mueller-Hinton agar (MHA, Difco™, NJ, USA) and incubated at 37°C overnight. Single colony was obtained from the agar plate and transferred into normal saline solution (NSS, 0.9% (w/v) NaCl). The turbidity of cell suspension was adjusted to 0.5 McFarland, which referred to approximately 10^8 CFU/ml were contained. The cell suspension was adjusted to 10^7 CFU/ml by 10 fold diluted of NSS

and inoculated to the MHA plates contained difference antimicrobial concentrations by using multipoint inoculator (Sigma-Aldrich®, MO, USA). The final inoculum on the plates were 10^4 CFU/spot approximately. The inoculated plates were left at room temperature until the inoculum was completely absorbed into the medium, then incubated at 37°C for 16-20 h. The MIC values were recorded as the lowest concentration of antimicrobial agents that colony formation was absented.

Table 2: Solvent, diluent and breakpoint of antimicrobial agents used in this study

Antimicrobial agent	Solvent	Diluent	MIC breakpoint (µg/ml)
amikacin	sterile distilled water	sterile distilled water	64
aztreonam	0.1 M $NaHCO_3$	sterile distilled water	32
carbenicillin	sterile distilled water	sterile distilled water	64
ceftazidime	10% (w/v) Na_2CO_3	sterile distilled water	32
chloramphenicol	95% ethanol	sterile distilled water	32
ciprofloxacin	sterile distilled water	sterile distilled water	4
erythromycin	95% ethanol	sterile distilled water	8
gentamicin	sterile distilled water	sterile distilled water	16*, 8**
kanamycin	sterile distilled water	sterile distilled water	64
neomycin	sterile distilled water	sterile distilled water	32
piperacillin	sterile distilled water	sterile distilled water	128
spectinomycin	sterile distilled water	sterile distilled water	128
streptomycin	sterile distilled water	sterile distilled water	128
tetracycline	sterile distilled water	sterile distilled water	16
trimethoprim	0.05 M HCl	sterile distilled water	4

* gentamicin breakpoint for human isolates

** gentamicin breakpoint for animal isolates

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3.2.2 Determination of the effect of efflux pump inhibitors on antimicrobial susceptibility

The effect of efflux pump inhibitors (EPIs) on antimicrobial susceptibility was examined by determination of the MIC of 15 antimicrobial agents as described above in the presence and absence of reserpine and carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) (n=130). Reserpine is an alkaloid derived from plants, which inhibit both primary and secondary active transporter systems as a competitive inhibitor of active efflux systems (Kumar et al., 2013). CCCP is a proton motive force inhibitor that inhibits secondary active transporters only (Ikonomidis et al., 2008). Reserpine and CCCP were purchased from Sigma-Aldrich® (MO, USA). The *A. baumannii* clinical isolates from humans and animals with difference antimicrobial resistance profile (n=45) were randomly selected for determination the MICs of reserpine and CCCP to select the concentration used and ensure no lethal effects to the cells. The experiment was carried out in duplicate of 2-fold agar dilution method under the CLSI guideline (CLSI, 2010). The antimicrobial susceptibility tests were performed using 2-fold agar dilution method according to CLSI (2010) as described in 3.2.1 with indicated concentrations of reserpine (25 µg/ml) and CCCP (5 µg/ml). Reduction of the MIC values were considered significant when at least 4-fold decreased was observed after reserpine or CCCP were added.

3.2.3 Determination of expression pattern of the RND multidrug efflux systems

Expression of the RND multidrug efflux systems including AdeABC, AdeFGH, AdeIJK and AdeDE was determined by using Reverse-Transcription PCR (RT-PCR) in all of the human and animal clinical isolates (n=130). AdeABC, AdeFGH, AdeIJK and AdeDE were encoded by *adeABC*, *adeFGH*, *adeIJK* and *adeDE* operons respectively. The inner membrane protein of each efflux system was encoded by *adeB*, *adeG*, *adeJ* and *adeE*. Expression of *adeB*, *adeG*, *adeJ* and *adeE* was detected to generate the expression pattern of RND efflux systems.

3.2.3.1 RNA extraction

The *A. baumannii* human and animal clinical isolates from -80°C stock were growth on LB plates at 37°C overnight. Single colony of the isolates were inoculated into LB broth, incubated at 37°C and harvested during the late log phase of growth for total RNA extraction by Total RNA Extraction Kit Mini (RBCBioscience, New Taipei City, Taiwan). On the following day, 1 ml of bacterial culture was transferred to 1.5 ml microcentrifuge tube, centrifuge at 13,000 rpm for 1 min. supernatant was discarded and the cell pellet was vortexed for 30 s. The cell pellet was added by 200 µl of RT buffer, the tube was vortexed and incubated at room temperature for 5 min. two hundred µl of RB buffer was added to the cell pellet and the cell suspension was incubated at room temperature for 5 min to lyse the cells. The cell lysate was added to a Filter Column Set and the column set was centrifuged at 13,000 rpm for 2 min. The clarified filtrate was added to a new 1.5 microcentrifuge tube and the Filter

Column Set was discarded. The filtrate was added by 300 µl of 70% ethanol and mixed by pipetting. The ethanol-added mixture was transferred to a RB Column Set and centrifugation of the column set was done at 13,000 rpm for 2 min, flow through was discarded. The RB column was washed with 400 µl of R-W1 buffer and centrifuged at 13,000 rpm for 1 min and flow through was discarded. Then, 600 µl of R-Wash buffer was added to the RB column and centrifuged at 13,000 rpm for 1 min, flow through was discarded. Further centrifugation was done at 13,000 rpm for 3 min to dry the RB column. The RB column was placed to new microcentrifuge tube and a 50 µl of RNase free water was added to the center of the column matrix. The tube with RB column was incubated at room temperature for 3 min. elution of RNA was done by final centrifuged at 13,000 rpm for 1 min. The extracted RNA concentration was determined by using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) at wavelengths of 260 and 280 nm. The extracted RNA was treated with DNase I (Thermo Fisher Scientific, MA, USA) to remove any remaining DNA in the RNA sample as described by manufacturer's instructions. One µg of RNA was mixed with 1 µl of 10X Reaction Buffer with $MgCl_2$ (100 mM Tris-HCl, 25 mM $MgCl_2$, 1 mM $CaCl_2$), 1 µl of 1 U/µl DNase I (50 mM Tris-HCl, 10 mM $Cacl_2$ and 50% v/v glycerol) and nuclease-free water was added to reach a 10 µl final volume reaction and the reaction mixture was incubated at 37°C for 30 min. The mixture was added by 1 µl of 50 mM EDTA and further incubation was done at 65°C for 10 min to stop the DNase I activity. The DNase treated RNA was stored at -20°C until used.

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3.2.3.2 cDNA synthesis and Reverse-Transcription PCR (RT-PCR)

Synthesization of cDNA was carried out by ImProm-II™ Reverse Transcriptase (Promega, WI, USA). The reverse transcription reaction was performed in a final volume 20 µl reaction. First, the 5 µl of 100 ng/µl DNase treated RNA and 1 µl of 10 µM reverse primer of each gene were mixed, then incubated at 70°C for 5 min and quick-chill on ice for 5 min. After that, 4 µl of ImProm-II™ 5X Reaction Buffer (250 mM Tris-HCl, 375 mM KCl and 50 mM DTT), 2 µl of 25 mM $MgCl_2$, 1 µl of dNTP mix (10 mM each dNTP), 7 µl of nuclease-free water and 1 µl of ImProm-II™ Reverse Transcriptase were added to the mixture. The reverse transcription steps were performed at 25°C for 5 min, 45°C for 45 min and 70°C for 15 min by thermocycler (Tpersonal combi model®, Biometra, Göttingen, Germany). The mixture excluding ImProm-II™ Reverse Transcriptase for each sample was set as control reaction (RT-). The synthesized cDNA was stored at -20°C until used.

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Detection of expression of RND multidrug efflux systems was performed using conventional polymerase chain reaction (PCR) with 50 µl reaction mixture containing 25 µl of 2x KAPA Taq ReadyMix (Kapa Biosystems, MA, USA), 2.5 µl of 10 µM forward primer, 2.5 µl of 10 µM reverse primer, 18 µl of nuclease-free water, 2 µl of the synthesized cDNA (25 ng/µl) and 2.5 µl of 10 µM following primer pairs: for *adeB*, AdeB_Fw_P5 and AdeB_Rv_P6; for *adeG*, AdeG_Fw_P7 and AdeG_Rv_P8; for *adeJ*, AdeJ_Fw_P9 and AdeJ_Rv_P10; and for *adeE*, AdeE_Fw_P11 and AdeE_Rv_P12 (Table 1 and Figure 4. The cycle conditions were performed as following, a cycle of 94°C for

5 min followed by 30 cycles of 94°C for 45 s, 61-65°C for 45°C, 72°C for 1 min and a cycle of 72°C for 5 min. annealing temperature (61-65°C) was varied depend on the detected genes. The RT-PCR products were analyzed on 1.5% agarose gel electrophoresis and visualized by Bio-Rad Gel Documentation system (Bio-Rad Laboratories, CA, USA) with ethidium bromide staining.

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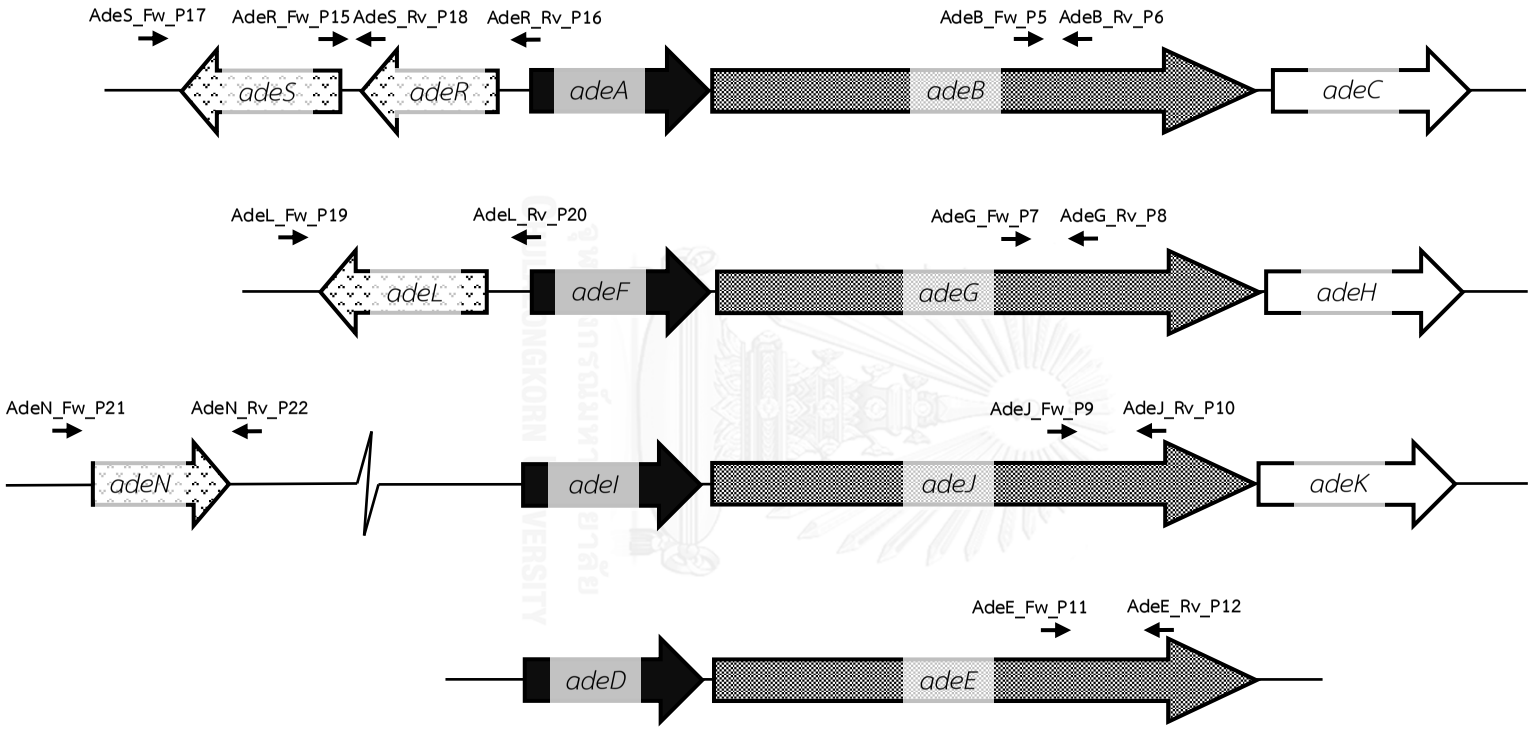

Figure 4: Schematic representation of the *adeABC*, *adeFGH, adeIJK* and *adeDE* operons and their regulatory genes. Open arrow indicate coding sequences and direction of transcription. Black arrows indicate the orientation and position of the primers used.

3.3 Characterization of expression and regulation of the RND multidrug efflux systems

Ten human clinical isolates (ABJ058, ABJ061, ABJ068, ABJ099, ABJ111, ABJ121, ABJ162, ABJ178, ABJ203 and ABJ208), which cover all of 5 expression patterns and 15 animal clinical isolates (ABJ302, ABJ303, ABJ308, ABJ311, ABJ316, ABJ317, ABJ319-321, ABJ323-325, ABJ328, ABJ329 and ABJ331) that cover all of 3 expression patterns were selected to determine the expression level of the RND efflux systems and characterize the regulatory genes. Determination of the expression level of efflux systems, including AdeABC, AdeFGH and AdeIJK was carried out by quantitative real-time reverse transcription PCR (qRT-PCR). Regulatory genes of each efflux system including, *adeRS*, *adeL* and *adeN* that regulated the efflux systems AdeABC, AdeFGH and AdeIJK, respectively, were screened for mutations by using conventional PCR and DNA sequencing.

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3.3.1 Determination of the expression level of RND multidrug efflux systems

cDNA of each gene from the selected isolates from 3.2.3 was 10-fold diluted by nuclease-free water to make cDNA template. The real-time qRT-PCR was conducted using KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, MA, USA) following the manufacturer's instructions. The total 20 µl qRT-PCR reaction mixture consisting of 10 µl of 2X KAPA SYBR® FAST qPCR Master Mix Universal, 1 µl of 10 µM of each forward and reverse primer, 3 µl of nuclease-free water and 5 µl of cDNA template. Real-time

qRT-PCR assays were carried out in Rotor-Gene™ 3000 (Corbett Research, Sydney, Australia) with the following step: enzyme activation at 95°C for 10 min followed by 40 cycles of 95°C for 45 s, 61°C for *adeB* and *adeG* or 62°C for *adeJ* and 65°C for 30 s, then dissociation at 60-95°C for 45 s in the first and hold for 5 s on the next steps. Homogeneity of the PCR product was determined by melting curves analysis. Each experiment was performed in triplicate and *rpsL* was used as internal control. The average cDNA copy number of *adeB*, *adeG* and *adeJ* was calculated from the Ct values from 2 independent experiments (SD<0.1). The standard curve (r^2 > 0.99) of *A. baumannii* ATCC19606 was generated by plotting the average Ct value against the log of five 10-fold serial dilution of cDNA template. The transcription levels more than 3-fold found in ATCC19606 were considered significant (Islam et al., 2004).

3.3.2 Characterization the regulatory genes

3.3.2.1 Chromosomal DNA extraction

Chromosomal DNA of the selected isolates was extracted by using QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) as following manufacturer's instructions. The isolates from -80°C were growth on LB agar and incubated at 37°C overnight. A single colony of the isolates was inoculated into LB broth and incubated in shaking incubator at 37°C overnight. On the next day, 1 ml of bacterial culture was transferred to 1.5 microcentrifuge tube and centrifuged at 7,500 rpm for 5 min. Supernatant was discarded and cell pellet was resuspended by add Buffer ATL to a total volume of 180 µl and the tube was vortexed. Twenty µl of proteinase K was added to the mixture

and mixed by vortex. The mixture was incubated at 56°C for 2 h, while the tube was vortexed 2-3 times/h during incubation. The lysate was added by 200 µl of Buffer AL and the tube was mixed by pulse-vortex for 15 s and further incubation at 70°C for 10 min. The sample was added by 200 µl of absolute ethanol and the tube was mixed by pulse-vortex for 15 s. The mixture was transferred to the QIAamp Mini Spin Column in a 2 ml collection tube and the column set was centrifuged at 8,000 rpm for 1 min. The silica membrane was placed into a new 2 ml collection tube and the old collection tube with filtrate was discarded. Five hundred µl of Buffer AW1 was added to the silica membrane and the column set was centrifuged at 8000 rpm for 1 min and the filtrate was discarded. The column set was added by 500 µl of Buffer AW2 and centrifuged at 14,000 rpm for 1 min. The silica membrane was placed into a new 1.5 ml microcentrifuge tube and the chromosomal DNA was eluted by 200 µl of Buffer AE. Incubation of the column set was done at room temperature for 5 min and final centrifugation was done at 8,000 rpm for 1 min. The extracted chromosomal DNA was stored at -20°C until used.

3.3.2.2 Conventional polymerase chain reaction (PCR)

The regulatory genes were amplified by using Tpersonal combi model® (Biometra, Göttingen, Germany) with KAPA Taq ReadyMix PCR Kit (Kapa Biosystems, MA, USA). two and a half µl of 10 µM following primer pairs: for *adeR*, AdeR_Fw_P15 and AdeR_Rv_P16; for *ade*S, AdeS_Fw_P17 and AdeS_Rv_P18; for *adeL*, AdeL_Fw_P19 and AdeL_Rv_P20; and for *adeN*, AdeN_Fw_P21 and AdeN_Rv_P22 (Table 1) was mixed

with 25 µl of 2x KAPA Taq ReadyMix, 2 µl of the genomic DNA templates (10-200 ng) and nuclease-free water was added to reach the total 50 µl reaction. The PCR cycling was consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, 52-58°C (depend on the regulatory genes) for 45 s, 72°C for 90 s and a final extension at 72°C for 5 min. PCR products were analyzed by Owl™ EasyCast™ B2 Mini Gel Electrophoresis Systems (Thermo Fisher Scientific, MA, USA) on 1.5% w/v agarose gel with 1x TAE buffer. The agarose gel was visualized by ethidium bromide stained in Bio-Rad Gel Documentation system (Bio-Rad Laboratories, CA, USA). The PCR products were purified by Nucleospin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and submitted to First BASE Laboratories (Selangor, Malaysia) for sequencing. Whole gene sequences obtained of each gene was compared to the sequence of *A. baumannii* reference strain ATCC19606 in the GenBank® database.

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3.4 Characterization of the contribution of multidrug efflux systems in biocide-antibiotic cross-resistance

3.4.1 *In vitro* exposure experiment

Three biocides included in this study were benzalkonium chloride, chlorhexidine and triclosan. The MIC values for antibiotics and all three biocides were determined using two-fold agar dilution according to CLSI guideline (CLSI, 2010) as described above. The human clinical isolates (n=10) and animal clinical isolates (n=15) that were susceptible to most antibiotics and exhibit low MICs to the biocides tested

were selected (Table 3). The *in vitro* exposure test procedure used in this study was applied from Gradel et al. (2005). The selected isolates were grown in 4 ml of LB broth and incubated at 37°C, overnight. On the following day, a 100 µl of inoculum was transferred to 4 ml of LB broth contained the biocides at the concentration 0.25-fold of the individual MIC values, incubate at 37°C overnight. Then, a 100 µl of inoculum from the previous day was transferred into LB broth with increasing concentration of biocide by 1.5 factor in each consecutive day until the bacterial growth was not observed. An inoculum turbidity was examined optically and the presence of bacteria in the inoculum was checked by spread on LB agar plates. The spontaneous mutant derivatives that grew in the medium with $\geq$4-fold concentration of the parental MICs were collected and stored at -80°C (Figure 5). The repetitive sequence-based PCR (rep-PCR) were performed to confirm the continuation of the parent and mutant derivatives (Woods et al., 1993). The rep-PCR reaction was used the primer pairs ERIC IR and ERIC II (Amonsin et al., 2003) for DNA amplification, which shown in Table 1. The PCR reaction was carried out by Tpersonal combi model® (Biometra, Göttingen, Germany) with initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 45°C for 5min, 72°C for 2 min and final extension at 72°C for 7 min. A total 50 µl PCR reaction mixture consisting of 25 µl of 2x KAPA Taq ReadyMix (Kapa Biosystems, MA, USA), 2.5 µl of each 10 µM primer, 18 µl of nuclease-free water and 2 µl of DNA template. The amplified DNA patterns were separated by Owl™ EasyCast™ B2 Mini Gel Electrophoresis Systems (Thermo Fisher Scientific, MA, USA) on 2% w/v agarose gel

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with 1x TAE buffer. The agarose gel was visualized by ethidium bromide stained in Bio-Rad Gel Documentation system (Bio-Rad Laboratories, CA, USA).

Table 3: Resistance phenotype and susceptibility of biocides in the selected isolates used in *in vitro* exposure experiment

Strains (n=25)	Resistance pattern	MIC of BZK (µg/ml)	MIC of CHG (µg/ml)	MIC of TCS (µg/ml)
ABJ058	ATM-CHL-ERY-PIP-SPE-STR-TET-TMP	32	64	0.5
ABJ106	AMK-ATM-CAR-CHL-ERY-GEN-KAN-NEO-PIP-SPE-TET-TMP	16	32	2
ABJ114	ATM-CHL-ERY-SPE-TMP	32	32	0.5
ABJ159	ATM-CHL-ERY-TMP	32	64	0.5
ABJ174	ATM-CHL-ERY-SPE-TET-TMP	32	8	0.5
ABJ178	ATM-CHL-ERY-SPE-TET-TMP	16	8	1
ABJ184	ATM-CAR-CAZ-CHL-ERY-SPE-STR-TMP	32	64	2
ABJ203	ATM-CAR-CAZ-CHL-ERY-TMP	32	32	2
ABJ205	ATM-CAR-CAZ-CHL-ERY-TET-TMP	16	16	1
ABJ208	ATM-CAR-CAZ-CHL-CIP-ERY-PIP-STR-TET-TMP	32	8	1
ABJ302	CHL	16	8	1
ABJ303	CHL-CIP-ERY-GEN-SPE-STR-TET-TMP	32	64	4
ABJ308	CHL-ERY-SPE-TMP	64	64	8
ABJ311	ATM-CHL-CIP-ERY-GEN-SPE-STR-TET-TMP	32	32	2
ABJ316	ATM-CHL-CIP-ERY-SPE-STR-TET-TMP	32	32	2
ABJ317	ATM-CHL-ERY-TMP	16	8	0.5
ABJ319	CAR-CHL-CIP-ERY-GEN-SPE-STR-TET-TMP	64	64	8
ABJ320	ATM-CAR-CHL-CIP-ERY-GEN-SPE-STR-TET-TMP	64	64	1
ABJ321	ATM-CAR-CHL-CIP-ERY-GEN-TET-TMP	32	16	2
ABJ323	AMK-ATM-CAR-CAZ-CHL-ERY-GEN-KAN-NEO-PIP-SPE-TMP	64	8	0.5
ABJ324	ATM-CHL-CIP-ERY-SPE-STR-TET-TMP	32	64	1
ABJ325	ATM-CHL-ERY-TMP	32	16	8
ABJ328	ATM-CHL-CIP-ERY-SPE-TMP	32	64	4
ABJ329	ATM-CHL-CIP-ERY-SPE-TET-TMP	32	16	1
ABJ331	ATM-CAR-CHL-CIP-ERY-GEN-PIP-SPE-STR-TET-TMP	32	64	1

AMK, amikacin; ATM, aztreonam; CAR, carbenicillin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; NEO, neomycin; PIP, piperacillin; SPE, spectinomycin; STR, streptomycin; TET, tetracycline; TMP, trimethoprim; BZK, benzalkonium chloride; CHG, chlorhexidine; TCS, triclosan.

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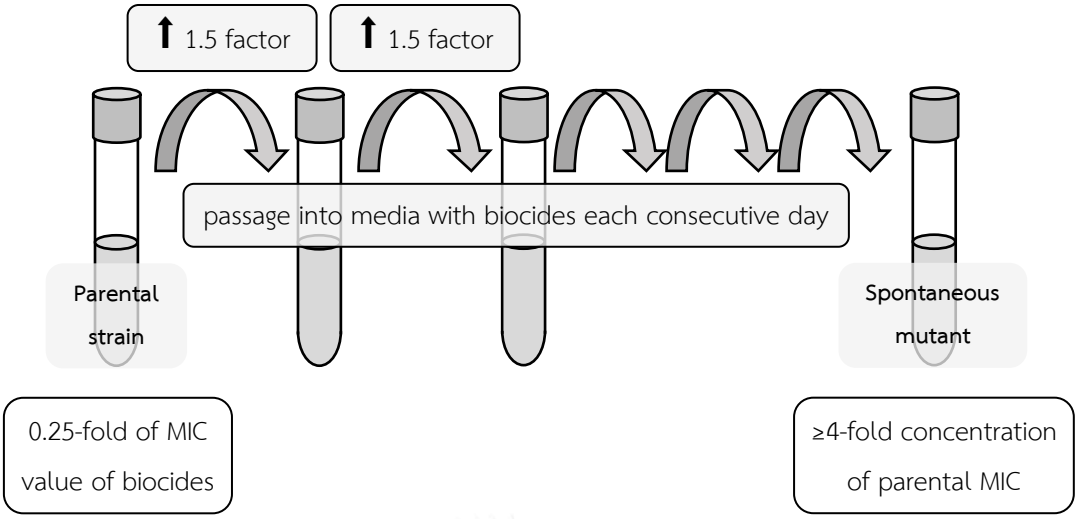

Figure 5: Schematic diagram of the *in vitro* exposure experiment procedure

3.4.2 Determination of MICs of antibiotics and biocides

MICs for antibiotics and the corresponding biocides in all of the mutant derivative strains from 3.4.1 were determined using two-fold agar dilution following the CLSI guideline (CLSI, 2010) as described in 3.2.1.

3.4.3 Detection of multidrug efflux systems expression

All of the mutant derivatives of human and animal isolates from 3.4.1 and their parental strains were tested for the expression of the multidrug efflux systems by using RT-PCR as described above in 3.2.3. The expression of the efflux system genes, including *adeB*, *adeG*, *adeJ*, *amvA* and *abeM* were determined and compared between mutant derivatives and their parental strains. The first three genes were the member of RND family, but the last two were the member of MFS and MATE efflux systems respectively. Analysis of the efflux pump expression in biocide-antibiotic cross-

resistance was carried out by detection the expression of *adeB*, *adeG*, *adeJ*, *amvA* and *abeM* between mutant derivatives and their parental strains.

3.4.3.1 RNA extraction

The mutant derivatives and their parental strains from -80°C stock were growth on LB plates at 37°C overnight. Single colony of the isolates were inoculated into LB broth, incubated at 37°C and the culture was harvested during the late log phase of growth for total RNA extraction by Total RNA Extraction Kit Mini (RBCBioscience, New Taipei City, Taiwan) as described in 3.2.3.1.

3.4.3.2 cDNA synthesis and Reverse-Transcription PCR (RT-PCR)

Synthesization of cDNA was carried out by ImProm-II™ Reverse Transcriptase (Promega, WI, USA). Reverse transcription protocol of AdeB, AdeG, adeJ, AmvA and AbeM was performed as described in 3.2.3.2 except for the cDNA of *amvA* and *abeM* was synthesized by using reverse primer, AmvA_Rv_P24 and AbeM_Rv_P26 (Table 1) instead of the reverse primer in 3.2.3.2. The PCR protocol was carried out for 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 45 s, 60/59°C (for AmvA/AbeM respectively) for 45 s, 65°C for 90 s and 1 cycle of 65°C for 5 min. The 2.5 µl of each 10 µM following primer pairs: for *amvA*, AmvA_Fw_P23 and AmvA_Rv_P24 and for *abeM*, AbeM_Fw_P25 and AbeM_Rv_P26 (Table 1) were added to a total 50 µl reaction containing 25 µl of 2x KAPA Taq ReadyMix (Kapa Biosystems, MA, USA), 18 µl of nuclease-free water and 2 µl of cDNA template. The amplified cDNA was separated by Owl™ EasyCast™ B2 Mini Gel Electrophoresis Systems (Thermo Fisher Scientific, MA, USA) on 1.5% w/v

agarose gel. The agarose gel was visualized by ethidium bromide stained in Bio-Rad Gel Documentation system (Bio-Rad Laboratories, CA, USA).

3.4.4 Determination of the expression level of RND multidrug efflux systems

Expression level of RND efflux pump, including AdeG and AdeJ in mutant derivatives and their parental strains was determined by using qRT-PCR as previously described in 3.3.1. Analysis of the RND efflux expression in biocide-antibiotic cross-resistance was carried out by compare the expression level of *adeG* and *adeJ* between mutant derivatives and their parental strains.

cDNA of the mutant derivatives and their parental strains from 3.4.3.2 was 10-fold diluted by nuclease-free water and used as the cDNA templates to determine the expression level of AdeG and AdeJ. Determination expression level of the genes was carried out by real-time qRT-PCR method as described in 3.3.1. The qRT-PCR was performed using KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, MA, USA) and the thermocycling was conducted in Rotor-Gene™ 3000 (Corbett Research, Sydney, Australia) as previously described in 3.3.1. *rpsL* was used as internal control and the qRT-PCR of each gene was done in triplicate. Calculation of average cDNA copy of the target genes was performed using the protocol as previously described in 3.3.1

3.4.5 Characterization the regulatory genes

A mutation in regulatory genes of efflux systems AdeFGH and AdeIJK was analyzed by compare the nucleotide sequence of *adeL* and *adeN* between mutant

derivatives and their parental strains. Amplification of the regulatory genes was carried out by conventional PCR as described in 3.3.2.2.

Extraction of chromosomal DNA of the mutant derivatives and their parental strains was performed using QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) as described in 3.2.3.1. Chromosomal DNA of the isolates was stored at-20℃ until used. Amplification of *adeL* and *adeN* was carried out by KAPA Taq ReadyMix PCR Kit (Kapa Biosystems, MA, USA) in Tpersonal combi model® (Biometra, Göttingen, Germany) as previously described in 3.3.2.2. The amplified DNA was separated by Owl™ EasyCast™ B2 Mini Gel Electrophoresis Systems (Thermo Fisher Scientific, MA, USA) in 1.5% w/v agarose gel. The agarose gel was stained by ethidium bromide and visualized in Bio-Rad Gel Documentation system (Bio-Rad Laboratories, CA, USA)

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CHAPTER IV

RESULTS

4.1 The *A. baumannii* clinical isolates

4.1.1 The *A. baumannii* human clinical isolates (n=100)

All the human isolates of *Acinetobacter* (n=100) from the bacterial strain collection of Department of Microbiology, Faculty of medicine Siriraj hospital, Mahidol University were confirmed to be the *baumannii* species by ARDRA (Dijkshoorn et al., 2007). The restriction patterns of 16S rRNA PCR amplicon with *Alu*I (400, 220 and 190 bp), *Mbo*I (616, 328, 192 and 160 bp) and *Cfo*I (645, 410, 258 and 200 bp) are shown in figure 6.

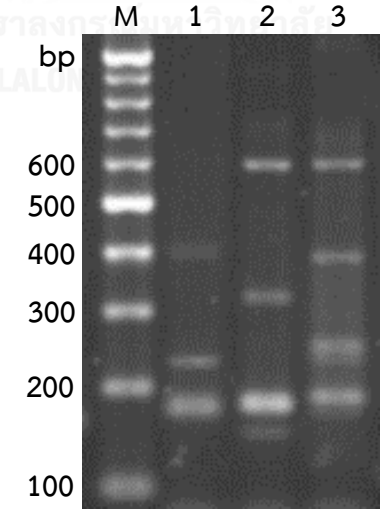

Figure 6: The restriction pattern of PCR-amplified 16S rRNA gene. Lane M, 100 bp DNA marker; Lane 1, *Alu*I; Lane 2, *Mbo*I; and Lane 3, *Cfo*I

4.1.2 The *A. baumannii* animal clinical isolates (n=30)

From 210 samples, 30 samples were positive to *A. baumannii* (14.29%). Thirty *A. baumannii* isolates were collected (one colony from each positive sample) and used for further study. The *A. baumannii* animal isolates were obtained from dogs (17/30, 56.67%), cats (8/30, 26.67%), rabbits (4/30, 13.33%) and pigs (1/30, 3.33%) (Table 4). For each animal species, the percentage of *A. baumannii* in dogs (n=112), cats (n=45), rabbits (n=17) and pigs (n=7) was 15.18%, 17.78%, 23.53% and 14.29% respectively.

Table 4: The presence of *A. baumannii* in the animal samples (n=210)

Species	No. of samples (n)	No. of *A. baumannii* positive samples (n, %)
Dog	112	17 (15.18)
Cat	45	8 (17.77)
Rabbit	17	4 (23.53)
Bird	15	0 (0)
Pig	7	1 (14.29)
Others[a]	14	0 (0)
Total	210	30 (14.29)

[a]Others; hedgehog (n=4), lizard (n=3), monkey (n=2), hamster (n=3), Gatsby (n=1) and chinchilla (n=1)

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4.2 Antimicrobial resistance phenotype

4.2.1 The *A. baumannii* clinical isolates from humans (n=100)

All *A. baumnannii* human clinical isolates (n=100) were resistant to at least four antimicrobial agents. All were resistant to erythromycin and trimethoprim (100%). The high resistance rates were observed for eight antimicrobial agents including chloramphenicol (99%), aztreonam (95%), spectinomycin (94%), ceftazidime (93%), tetracycline (93%), piperacillin (91%), carbenicillin (90%) and streptomycin (90%). The resistance rates for other antimicrobial agents including gentamicin, kanamycin, ciprofloxacin, amikacin and neomycin were 89%, 87%, 81% 78% and 73%, respectively. The distribution of antimicrobial resistance is shown in figure 7.

Ninety-eight percent of the human isolates were multidrug resistant (MDR) *A. baumannii* that defined by being resistant to at least 6 difference antimicrobial agents (Gu et al., 2007; Poonsuk et al., 2012). Antimicrobial resistance phenotype was grouped into 28 resistance patterns (Table 5). The predominant resistance pattern was AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP (53%), followed by AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-PIP-SPE-STR-TET-TMP (12%).

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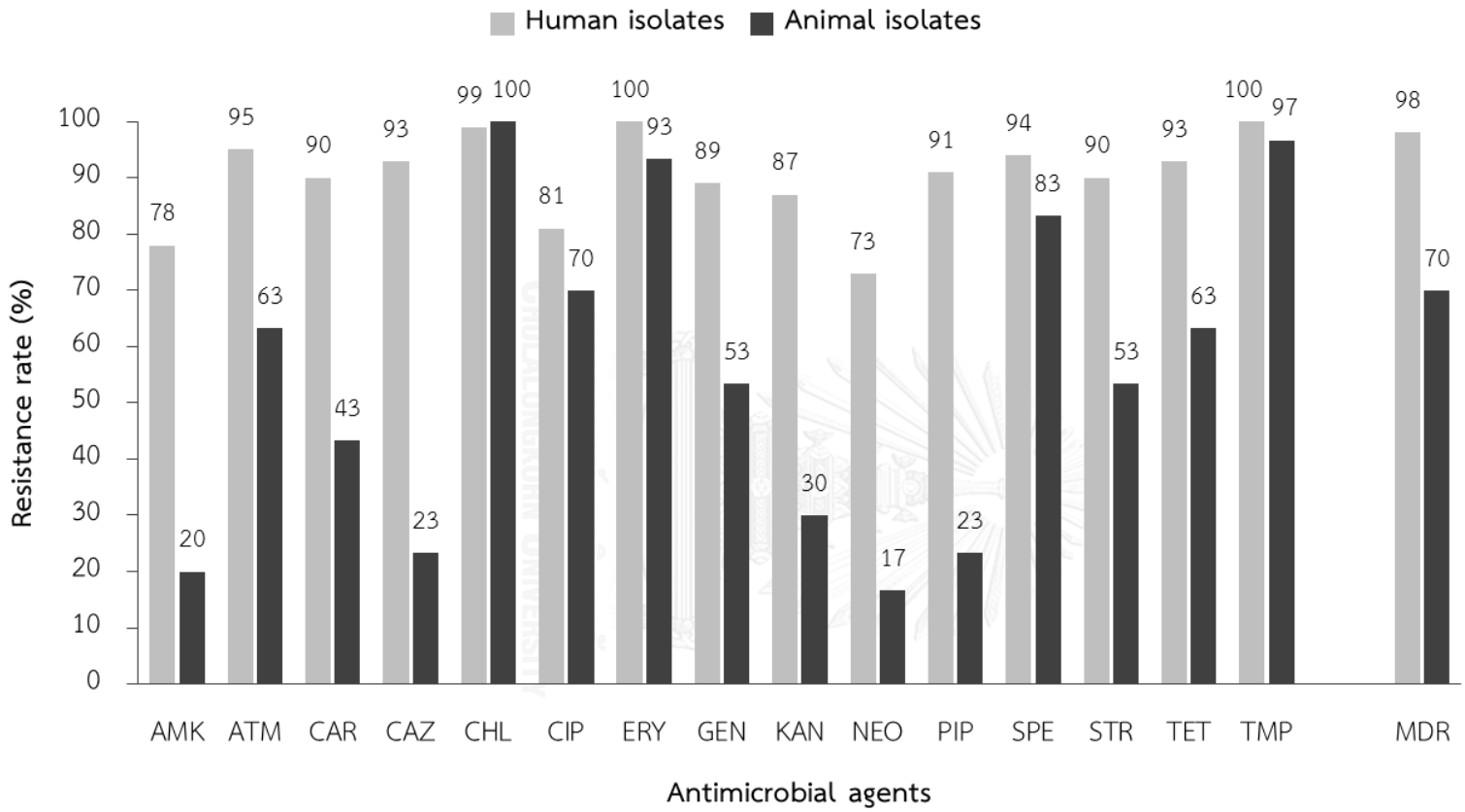

Figure 7: Distribution of antimicrobial resistance among the *A. baumannii* clinical isolates form humans (n=100) and animals (n=30). AMK, amikacin; ATM aztreonam; CAR, carbenicillin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; NEO, neomycin; PIP, piperacillin; SPE, spectinomycin; STR, streptomycin; TET, tetracycline; TMP, trimethoprim; MDR, multidrug resistance

Table 5: Antimicrobial resistance pattern of the *A. baumannii* human isolates (n=100)

Antimicrobial resistance pattern	No. of isolates (%)
ATM-CHL-ERY-TMP	1 (1%)
ATM-CHL-ERY-SPE-TMP	1 (1%)
ATM-CAR-CAZ-CHL-ERY-TMP	1 (1%)
ATM-CHL-ERY-SPE-TET-TMP	2 (2%)
ATM-CAR-CAZ-CHL-ERY-TET-TMP	1 (1%)
ATM-CAR-CAZ-CHL-ERY-SPE-STR-TMP	1 (1%)
ATM-CHL-ERY-PIP-SPE-STR-TET-TMP	1 (1%)
ATM-CHL-CIP-ERY-GEN-PIP-SPE-TET-TMP	1 (1%)
ATM-CAR-CAZ-CHL-CIP-ERY-PIP-STR-TET-TMP	1 (1%)
CAZ-CHL-ERY-GEN-KAN-PIP-SPE-STR-TET-TMP	1 (1%)
ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-TET-TMP	1 (1%)
CAZ-CHL-CIP-ERY-GEN-KAN-PIP-SPE-STR-TET-TMP	1 (1%)
CAZ-CHL-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP	1 (1%)
AMK-ATM-CAR-CHL-ERY-GEN-KAN-NEO-PIP-SPE-TET-TMP	1 (1%)
AMK-CAZ-CHL-CIP-ERY-GEN-NEO-PIP-SPE-STR-TET-TMP	1 (1%)
ATM-CAR-CAZ-CHL-CIP-ERY-GEN-PIP-SPE-STR-TET-TMP	2 (2%)
ATM-CAR-CAZ-CHL-CIP-ERY-KAN-NEO-PIP-SPE-STR-TMP	1 (1%)
AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-PIP-TET-TMP	1 (1%)
AMK-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-SPE-STR-TET-TMP	1 (1%)
ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-PIP-SPE-STR-TET-TMP	1 (1%)
ATM-CAR-CAZ-CHL-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP	3 (3%)
AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-PIP-SPE-STR-TMP	2 (2%)
AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-PIP-SPE-STR-TET-TMP	12 (12%)
AMK-ATM-CAR-CAZ-CHL-CIP-ERY-KAN-NEO-PIP-SPE-STR-TET-TMP	1 (1%)
AMK-ATM-CAR-CAZ-CHL-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP	5 (5%)
AMK-ATM-CAR-CAZ-CIP-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP	1 (1%)
ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP	1 (1%)
AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP	53 (53%)

4.2.2 The *A. baumannii* clinical isolates from animals (n=30)

All *A. baumnannii* animal clinical isolates (n=30) were resistant to at least one antimicrobial agent. All were resistant to chloramphenicol (100%). Most of the isolates were resistant to trimethoprim (97%), followed by erythromycin (93%), spectinomycin (83%), ciprofloxacin (70%), aztreonam and tetracycline (63%). Resistance rates for gentamicin, streptomycin, carbenicillin, kanamycin, ceftazidime, piperacillin, amikacin and neomycin were 53%, 53%, 43%, 30%, 23%, 23%, 20% and 17%, respectively (Figure 7).

The majority of the animal isolates (70%) were MDR and antimicrobial resistance phenotype was categorized into 23 patterns (Table 6). The most common resistance pattern was CHL-ERY-SPE-TMP (13%), followed by AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP (6.7%), ATM-CHL-CIP-ERY-SPE-STR-TET-TMP (6.7%), CHL-CIP-ERY-GEN-SPE-STR-TET-TMP (6.7%) and ATM-CHL-ERY-TMP (6.7%).

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Table 6: Antimicrobial resistance pattern of the *A. baumannii* animal isolates (n=30)

Antimicrobial resistance pattern	No. of isolates (%)
CHL	1 (3.3%)
ATM-CHL-ERY-TMP	2 (6.7%)
CHL-ERY-SPE-TMP	4 (13.3%)
ATM-CAR-CHL-ERY-TMP	1 (3.3%)
CHL-CIP-ERY-SPE-TMP	1 (3.3%)
ATM-CHL-CIP-ERY-SPE-TMP	1 (3.3%)
ATM-CHL-CIP-SPE-TET-TMP	1 (3.3%)
ATM-CHL-CIP-ERY-SPE-TET-TMP	1 (3.3%)
ATM-CAR-CHL-CIP-ERY-GEN-TET-TMP	1 (3.3%)
ATM-CHL-CIP-ERY-SPE-STR-TET-TMP	2 (6.7%)
CHL-CIP-ERY-GEN-SPE-STR-TET-TMP	2 (6.7%)
ATM-CHL-CIP-ERY-GEN-SPE-STR-TET-TMP	1 (3.3%)
CAR-CHL-CIP-ERY-GEN-SPE-STR-TET-TMP	1 (3.3%)
ATM-CAR-CHL-CIP-ERY-GEN-SPE-STR-TET-TMP	1 (3.3%)
CHL-CIP-ERY-GEN-KAN-NEO-SPE-STR-TET-TMP	1 (3.3%)
ATM-CAR-CHL-CIP-ERY-GEN-PIP-SPE-STR-TET-TMP	1 (3.3%)
AMK-ATM-CAR-CAZ-CHL-ERY-GEN-KAN-NEO-PIP-SPE-TMP	1 (3.3%)
AMK-ATM-CAR-CHL-CIP-ERY-GEN-KAN-SPE-STR-TET-TMP	1 (3.3%)
AMK-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-PIP-SPE-STR-TET-TMP	1 (3.3%)
ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-SPE-STR-TET-TMP	1 (3.3%)
ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-PIP-SPE-STR-TET-TMP	1 (3.3%)
AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-PIP-SPE-STR-TET-TMP	1 (3.3%)
AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP	2 (6.7%)

4.3 Effect of efflux pump inhibitors (reserpine and CCCP) on antimicrobial susceptibility

4.3.1 The *A. baumannii* clinical isolates from humans (n=100)

Overall, the addition of reserpine resulted in reduced resistance rates for 10 antimicrobial agents including neomycin (18%), tetracycline (15%), amikacin and gentamicin (14%), piperacillin (13%), ceftazidime and kanamycin (12%), aztreonam (9%), carbenicillin (8%) and streptomycin (6%) (the percentage of reduction is in parentheses). The presence of reserpine had no effect on resistance rates of five antimicrobial drugs including chloramphenicol, ciprofloxacin, erythromycin, spectinomycin and trimethoprim (Figure 8). When considered MIC level, reserpine reduced the MIC value of gentamicin, tetracycline and piperacillin by 4-fold in most human isolates (70%, 61% and 52% respectively). The effect of reserpine on the 4-fold reduction of MIC value of other antimicrobial agents varied between 4-42%, including ceftazidime (42%), neomycin (39%), streptomycin (28%), kanamycin (24%), aztreonam (21%), carbenicillin (20%), amikacin (19%), chloramphenicol (15%), spectinomycin (9%), trimethoprim (6%) and erythromycin (4%). No reduction effect was observed for ciprofloxacin (Table 7).

The presence of CCCP resulted in the reduction of resistance rates in all antimicrobial drugs tested except chloramphenicol and trimethoprim. The resistance rates of 13 antimicrobial agents reduced from 2 to 32% (i.e. amikacin (32%), neomycin (28%), gentamicin (21%), ceftazidime (14%), carbenicillin and piperacillin (11%),

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streptomycin and tetracycline (8%), kanamycin (7%), aztreonam (4%), ciprofloxacin (3%), erythromycin and spectinomycin (2%)) (Figure 8). The addition of CCCP reduced the MICs at least 4-fold in all of 15 antimicrobials tested at different percentage including gentamicin (60%), piperacillin (52%), tetracycline (50%), neomycin (46%), erythromycin (41%), amikacin and ceftazidime (38%), kanamycin (30%), streptomycin (26%), trimethoprim (25%), aztreonam (21%), chloramphenicol (18%), spectinomycin (14%), ciprofloxacin (8%) and carbenicillin (4%) (Table 7).

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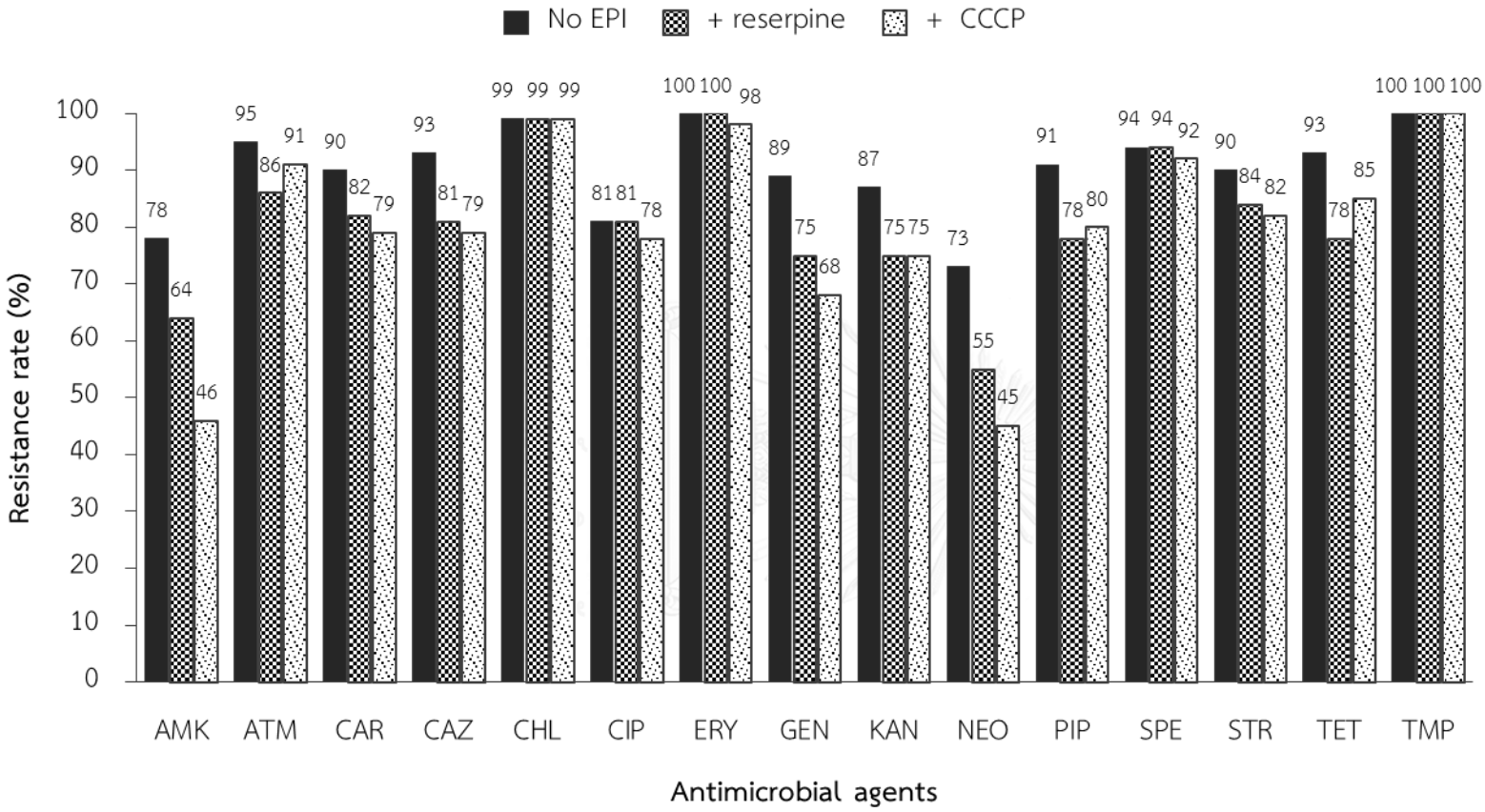

Figure 8: Distribution of antimicrobial resistance in the *A. baumannii* clinical isolates form humans (n=100). No EPI, antimicrobials without efflux pump inhibitor; + reserpine, antimicrobials with reserpine 25 µg/ml; + CCCP, antimicrobials with CCCP 5 µg/ml

Table 7: Effect of reserpine and CCCP on resistance rates and MIC of 15 antimicrobial agents in the *A. baumannii* human clinical isolates

	No EPI		+ Reserpine (25 µg/ml)			+ CCCP (5 µg/ml)		
Antimicrobial agent	Resistance rates (%)	MIC range (µg/ml)	Resistance rates (%)	MIC range (µg/ml)	No. of isolates (%) with ≥4-fold reduction	Resistance rates (%)	MIC range (µg/ml)	No. of isolates (%) with ≥4-fold reduction
amikacin	78	8 - >1024	64	4 - >1024	19 (19%)	46	4 - >1024	38 (38%)
aztreonam	95	16 - >256	86	8 - >256	21 (21%)	91	8 - >256	21 (21%)
carbenicillin	90	16 - >2048	82	16 - >2048	20 (20%)	79	16 - >2048	4 (4%)
ceftazidime	93	8 - >2048	81	8 - >2048	42 (42%)	79	4 - >2048	38 (38%)
chloramphenicol	99	16 - 512	99	8 - 512	15 (15%)	99	4 - 512	18 (18%)
ciprofloxacin	81	0.25 - 256	81	0.25 - 256	-	78	≤0.0625 - 128	8 (8%)
erythromycin	100	64 - 1024	100	64 - 512	4 (4%)	98	4 - 512	41 (41%)
gentamicin	89	2 - >2048	75	2 - >512	70 (70%)	68	0.5 - >512	60 (60%)
kanamycin	87	2 - >2048	75	2 - >2048	24 (24%)	75	2 - >2048	30 (30%)
neomycin	73	8 - >2048	55	4 - >2048	39 (39%)	45	4 - >2048	46 (46%)
piperacillin	91	32 - >1024	78	16 - >1024	52 (52%)	80	16 - >1024	52 (52%)
spectinomycin	94	64 - >2048	94	64 - >2048	9 (9%)	92	64 - 2048	14 (14%)
streptomycin	90	16 - >2048	84	16 - >2048	28 (28%)	82	16 - >2048	26 (26%)
tetracycline	93	0.25 - 2048	78	0.25 - 512	61 (61%)	85	0.25 - 512	50 (50%)
trimethoprim	100	16 - >1024	100	16 - >1024	6 (6%)	100	16 - >1024	25 (25%)

4.3.2 The *A. baumannii* clinical isolates from animals (n=30)

Of all antimicrobial agents tested the presence of reserpine resulted in the reduction of resistance rates for eight drugs including aztreonam (26%); spectinomycin (23%); carbenicillin (13%); neomycin and tetracycline (10%); amikacin, ceftazidime and erythromycin (3%) (Figure 9). Reserpine caused 4-fold reduction of MIC for seven antimicrobial agents including neomycin and tetracycline (4%); carbenicillin and ceftazidime (2%); erythromycin, spectinomycin and trimethoprim (1%).

The addition of CCCP decreased the resistance rates of eight antimicrobial agents from 3-26% (i.e. spectinomycin (26%); aztreonam (20%); carbenicillin (13%); amikacin and neomycin (10%); erythromycin (6%); ceftazidime and chloramphenicol (3%)). The presence of CCCP caused 4-fold reduction in all antimicrobials tested except in aztreonam and ciprofloxacin. The effect of CCCP resistance rates and MIC of 15 antimicrobial agents in the *A. baumannii* animal clinical is shown in table 8, These included neomycin (40%); chloramphenicol (13%); erythromycin, streptomycin, tetracycline and trimethoprim (10%); amikacin and piperacillin (6.7%); carbenicillin, ceftazidime, gentamicin, kanamycin and spectinomycin (3.3%).

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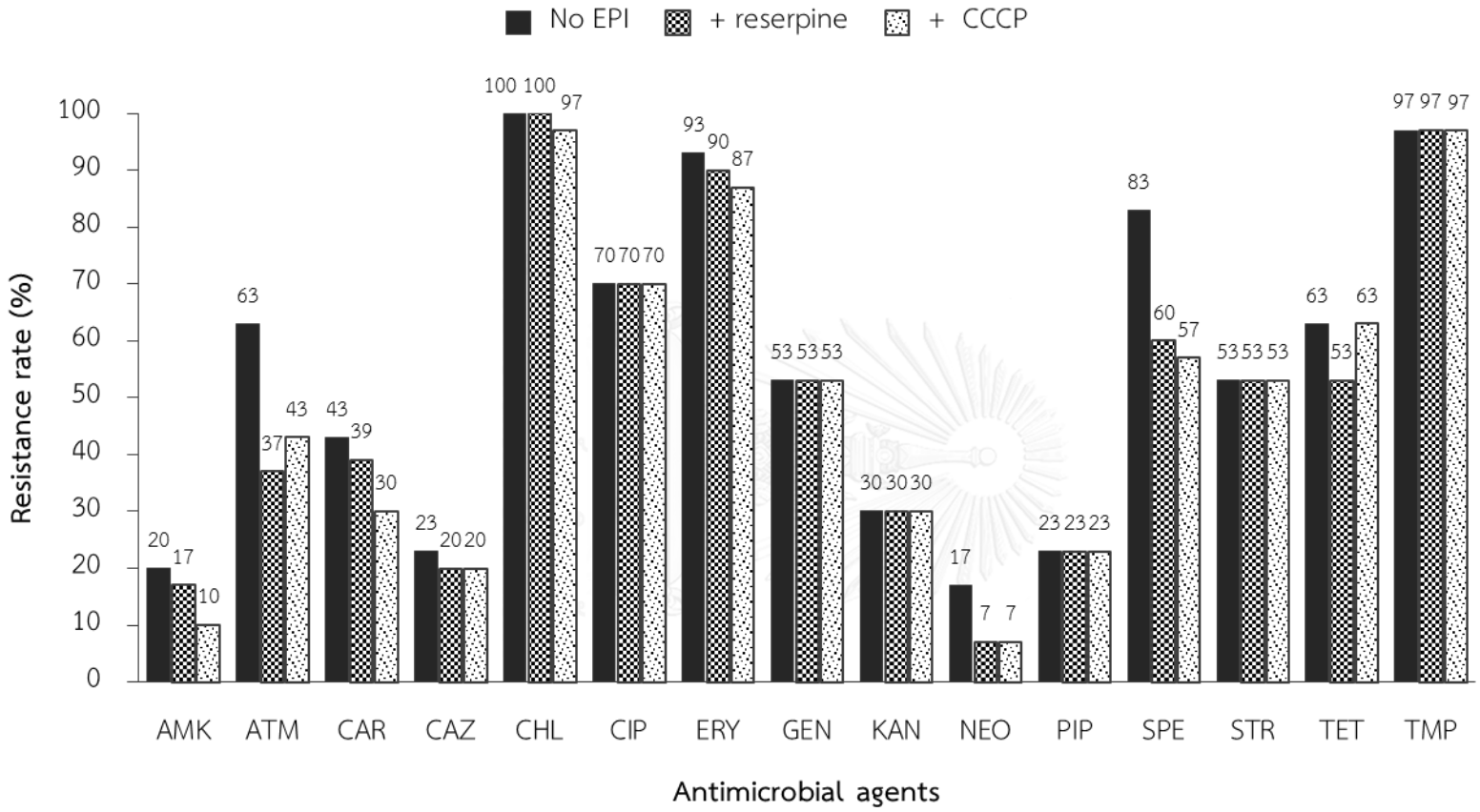

Figure 9: Distribution of antimicrobial resistance in the *A. baumannii* clinical isolates form animals (n=100). No EPI, antimicrobials without efflux pump inhibitor; + reserpine, antimicrobials with reserpine 25 µg/ml; + CCCP, antimicrobials with CCCP 5 µg/ml

Table 8: Effect of reserpine and CCCP on resistance rates and MIC of 15 antimicrobial agents in the *A. baumannii* animal clinical isolates

	No EPI		+ Reserpine (25 µg/ml)			+ CCCP (5 µg/ml)		
Antimicrobial agent	Resistance rates (%)	MIC range (µg/ml)	Resistance rates (%)	MIC range (µg/ml)	No. of isolates (%) with ≥4-fold reduction	Resistance rates (%)	MIC range (µg/ml)	No. of isolates (%) with ≥4-fold reduction
amikacin	20	1 - 128	17	1 - 64	-	10	1 - 64	2 (6.7%)
aztreonam	63	8 - >256	37	4 - >256	-	43	8 - >256	-
carbenicillin	43	8 - >1024	30	8 - >1024	2 (6.7%)	30	4 - >1024	1 (3.3%)
ceftazidime	23	0.5 - >256	20	0.5 - >256	2 (6.7%)	20	0.5 - >256	1 (3.3%)
chloramphenicol	100	32 - >256	100	32 - >256	-	97	16 - >256	4 (13%)
ciprofloxacin	70	0.03 - >64	70	0.03 - >64	-	70	0.03 - >64	-
erythromycin	93	0.5 - >128	90	0.5 - >128	1 (3.3%)	87	0.5 - >128	3 (10%)
gentamicin	53	0.5 - >128	53	0.5 - >128	-	53	0.5 - >128	1 (3.3%)
kanamycin	30	1 - >512	30	1 - >512	-	30	1 - >512	1 (3.3%)
neomycin	17	1 - 128	7	1 - 64	4 (13%)	7	1 - 32	12 (40%)
piperacillin	23	2 - >512	23	2 - >512	-	23	2 - >512	2 (6.7%)
spectinomycin	83	32 - >1024	60	32 - >1024	1 (3.3%)	57	8 - >1024	1 (3.3%)
streptomycin	53	4 - >1024	53	4 - >1024	-	53	2 - >1024	3 (10%)
tetracycline	63	0.5 - >256	53	0.5 - >256	4 (13%)	63	0.5 - >256	3 (10%)
trimethoprim	97	1 - >128	97	1 - >128	1 (3.3%)	97	1 - >128	3 (10%)

4.4 Distribution of RND multidrug efflux systems

All the *A. baumannii* clinical isolates from humans (n=100) and animals (n=30) were examined for the expression of RND efflux pump (AdeABC, AdeFGH, AdeIJK and AdeDE) by RT-PCR. The PCR amplicon size of the RND transporters including *adeB*, *adeG*, *adeJ* and *adeE* was 153 bp, 245 bp, 346 bp and 460 bp respectively (Figure 10).

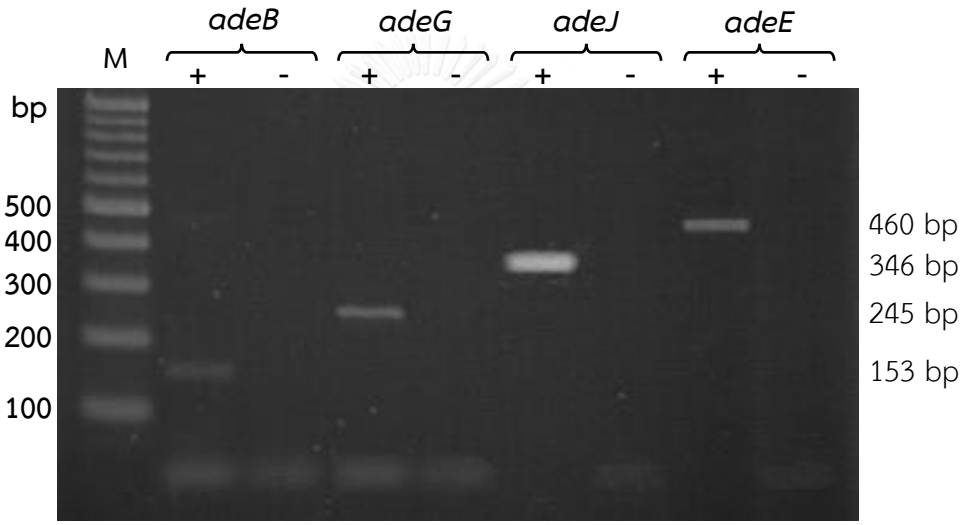

Figure 10: RT-PCR result of *adeB*, *adeG*, *adeJ* and *adeE*. Lane M, 100 bp DNA marker; +, with reverse transcriptase and -, without reverse transcriptase

4.4.1 The RND efflux pumps in the *A. baumannii* clinical isolates from humans (n=100)

Of all *A. baumannii* human clinical isolates, the isolates that expressed of *adeB*, *adeG* and *adeJ* were 83%, 61% and 97%, respectively. None of the isolates expressed *adeE* as detected by RT-PCR (Figure 11)

Based on the expression of the RND efflux pumps tested, five expression patterns were identified (Table 9). The expression pattern, AdeB-AdeG-AdeJ, was found in most human isolates (52%), followed by AdeB-AdeJ (31%). Nine percent of the isolates expressed AdeG and AdeJ (AdeG-AdeJ), simultaneously. Five isolates (5%) expressed only AdeJ. The expression of RND efflux systems was not observed in three isolates (3%).

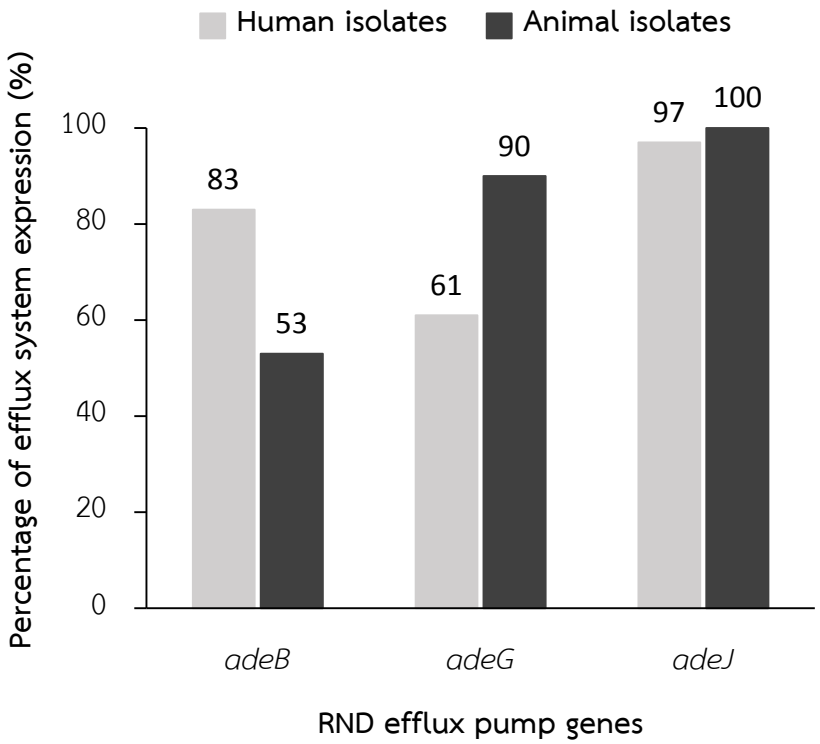

Figure 11: Distribution of the expressing RND efflux systems among the *A. baumannii* clinical isolates form humans (n=100) and animals (n=30)

Table 9: Distribution of expression pattern of the RND efflux pumps in the *A. baumannii* clinical isolates from humans (n=100)

Expression pattern	No. of isolates (%)
None	3 (3%)
AdeJ	5 (5%)
AdeB-AdeJ	31 (31%)
AdeG-AdeJ	9 (9%)
AdeB-AdeG-AdeJ	52 (52%)
Total	100 (100%)

4.4.2 The RND efflux pumps in the *A. baumannii* clinical isolates from animals (n=30)

All the *A. baumannii* animal isolates expressed *adeJ* (100%). Of the 30 animal isolates, transcription of *adeB* and *adeG* were positive was observed in 16 (53%) and 27 (90%) isolates, respectively. None of the animal isolates were positive for *adeE* expression (Figure 11).

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The expression patterns of the RND efflux pump obtained from *A. baumannii* clinical isolates from animals were AdeB-AdeG-AdeJ (53%), AdeG-AdeJ (37%) and AdeJ (10%) (Table 10).

Table 10: Distribution of expression pattern of the RND efflux pumps in the *A. baumannii* clinical isolates from animals (n=30)

Expression pattern	No. of isolates (%)
AdeJ	3 (10%)
AdeG-AdeJ	11 (37%)
AdeB-AdeG-AdeJ	16 (53%)
Total	30 (100%)

4.5 Transcription level of RND multidrug efflux systems

4.5.1 The *A. baumannii* clinical isolates from humans (n=10)

The expression level of RND efflux systems, mutation of corresponding regulatory and antimicrobial resistance patterns of the selected *A. baumannii* human clinical isolates are shown in table 11. The main findings of the expression level of the RND multidrug efflux systems in the human isolates were as follows:

No transcription of *adeB* was detected in all the *A. baumannii* human clinical isolates tested, in comparison to *A. baumannii* ATCC19606. These included ABJ111 and ABJ121, which were resistant to all 15 antimicrobial drugs tested.

The expression level of *adeG* of the human isolates was 0.3 to 4.5 fold. The overexpression of *adeG* was detected in two human isolates and both additionally expressed *adeJ* confirmed by RT-PCR. The highest expression level of *adeG* (4.5 fold) was detected in ABJ162 that was resistant to all antimicrobials tested. The isolate ABJ178 that was resistant to six antimicrobial tested expressed 3 fold of *adeG*.

The expression level of *adeJ* was 0.4 to 5 fold. The highest expression level of *adeJ* (5 fold) was found in ABJ162 that also expressed *adeG* at highest level. The expression level of *adeJ* in the other isolates varied from 0.4 to 2 fold and these isolates were resistant to at least six antimicrobial agents tested.

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Table 11: Expression of the RND efflux systems, mutation in the corresponding regulatory regions and antimicrobial resistance pattern in the *A. baumannii* clinical isolates from humans (n=10)

Expression pattern	Strain	Transcription level[a]			Regulatory mutation[b]				Antimicrobial resistance pattern
		adeB	*adeG*	*adeJ*	AdeR	AdeS	AdeL	AdeN	
I	ABJ061	-	-	-	Val120-Ile, Ala136-Val, Pro241-Leu	Ala153-Thr, Gly186-Val, Leu214-Phe, Ser263-Ala, Ala280-Ser, Asp281-Gln, Val331-Ile, Val348-Ile	Arg2-Cys	Met197-Thr	AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP
	ABJ099	-	-	-	Val120-Ile, Ala136-Val, Pro241-Leu	Ala153-Thr, Asp167-Tyr, Gly168-Cys, Gly186-Val, Leu214-Phe, Ser263-Ala, Asn268-His, Ala280-Ser, Asp281-Gln, Val331-Ile, Val348-Ile	ND	Met197-Thr	AMK-ATM-CAR-CAZ-CHL-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP
II	ABJ068	-	-	0.5	ND	ND	-	Met174-Thr, Met197-Thr	AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP
	ABJ208	-	-	1.1	ND	ND	-	Met174-Thr, Met197-Thr	ATM-CAR-CAZ-CHL-CIP-ERY-PIP-STR-TET-TMP
III	ABJ058	0.1	-	0.4	Asn134-Lys, His195-Gln, Pro241-Leu	Iso73-Val, Ala153-Thr, Leu214-Phe, Val245-Ile, Ser263-Ala, Val279-Ala, Ala280-Ser, Asp281-Gln, Val331-Ile, Ser354-Pro	-	Met197-Thr	ATM-CHL-ERY-PIP-SPE-STR-TET-TMP
	ABJ111	0.2	-	1.4	Pro241-Leu	ND	Gln264-Arg	Gly177-Ter	AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP
IV	ABJ162	-	4.5	5.0	ND	ND	-	ND	AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP
	ABJ178	-	3.0	2.0	ND	Gly41-Ala, Trp42-Gly, Ile43-Leu, Ser44-Val, Leu45-Ter	-	Met197-Thr	ATM-CHL-ERY-SPE-TET-TMP
V	ABJ121	0.1	0.3	0.4	Pro241-Leu	ND	Asp227-Ala, Gln264-Arg	Met197-Thr	AMK-ATM-CAR-CAZ-CHL-CIP-ERY-GEN-KAN-NEO-PIP-SPE-STR-TET-TMP
	ABJ203	0.1	1.2	0.4	Val120-Ile, Ala133-Thr, Pro241-Leu, Val243-Ile	Ala153-Thr, Glu174-Lys, Ser341-Cys, Met355-Leu	-	Met197-Thr	ATM-CAR-CAZ-CHL-ERY-TMP

[a]-, no expression.

[b]-, no mutations found; ND, not detected.

4.5.2 The *A. baumannii* clinical isolates from animals (n=15)

The expression level of RND efflux systems, mutation of corresponding regulatory genes and antimicrobial resistance pattern of the selected *A. baumannii* animal clinical isolates are shown in table 12. The main findings of the expression level of the RND multidrug efflux systems in the animal isolates were as follows:

The transcription level of *adeB* in the selected *A. baumannii* clinical isolates from animals varied from 0.1 to 2.4 fold of the *A. baumannii* ATCC19606. Therefore, none of the animal isolates overexpressed *adeB* when 3 fold was used as criteria for overexpression.

The transcription level of *adeG* in the selected animal isolates was 0.1 fold to 214.8 fold. All the animal isolates overexpressing *adeG* (7.4 to 214.8 fold) except in ABJ317 (0.1 fold) and ABJ316 (2 fold). The isolates with *adeG* overexpression (7.4 to 214.8 fold) were resistant to at least one antimicrobial agent tested. The highest expression of *adeG* isolates was detected in ABJ328 (214.8 fold) that was resistant to six antimicrobial drugs.

The expression level of *adeJ* in the selected animal clinical isolates was 0.1 to 16.7 fold. All of the *adeJ* overexpressing isolates were additionally positive to *adeG* by RT-PCR. The lowest expression of *adeJ* (0.1 fold) was detected in two isolates, of which one isolate (ABJ323) was resistant to 12 antimicrobial drugs and the other (ABJ324) was resistant to eight antimicrobial drugs.

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Table 12: Expression of the RND efflux systems, mutation in the corresponding regulatory regions and antimicrobial resistance pattern in the *A. baumannii* clinical isolates from animals (n=15)

Expression pattern	Strain	Transcription level			Regulatory mutation		Antimicrobial resistance pattern
		adeB	*adeG*	*adeJ*	AdeL	AdeN	
I	ABJ308	-	-	6.6	-	Gln94-Arg	CHL-ERY-SPE-TMP
	ABJ323	-	-	0.1	-	ND	AMK-ATM-CAR-CAZ-CHL-ERY-GEN-KAN-NEO-PIP-SPE-TMP
	ABJ324	-	-	0.1	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr	ATM-CHL-CIP-ERY-SPE-STR-TET-TMP
II	ABJ302	-	108.4	11.6	Ile145-Val	Asn195-His, Met197-Thr	CHL
	ABJ317	-	0.1	8.0	-	ND	ATM-CHL-ERY-TMP
	ABJ325	-	42.0	6.8	-	ND	ATM-CHL-ERY-TMP
	ABJ328	-	214.8	8.4	-	Met197-Arg	ATM-CHL-CIP-ERY-SPE-TMP
	ABJ329	-	19.8	7.8	-	Val44dup	ATM-CHL-CIP-ERY-SPE-TET-TMP
III	ABJ303	2.4	19.6	6.3	-	-	CHL-CIP-ERY-GEN-SPE-STR-TET-TMP
	ABJ311	1.3	13.4	13.3	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr	ATM-CHL-CIP-ERY-GEN-SPE-STR-TET-TMP
	ABJ316	0.2	2.0	2.0	-	-	ATM-CHL-CIP-ERY-SPE-STR-TET-TMP
	ABJ319	2.4	7.4	14.1	-	Pro16-Ser, Met186-Ile, Met197-Thr	CAR-CHL-CIP-ERY-GEN-SPE-STR-TET-TMP
	ABJ320	2.2	33.4	14.0	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr	ATM-CAR-CHL-CIP-ERY-GEN-SPE-STR-TET-TMP
	ABJ321	0.1	62.3	16.7	-	Val44dup	ATM-CAR-CHL-CIP-ERY-GEN-TET-TMP
	ABJ331	2.2	10.3	2.1	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr	ATM-CAR-CHL-CIP-ERY-GEN-PIP-SPE-STR-TET-TMP

[a]-, no expression.

[b]-, no mutations found; ND, not detected.

4.6 Mutations in the RND efflux pump regulatory genes

Ten *A. baumannii* human clinical isolates and 15 *A. baumannii* animal clinical isolates from 4.5 were determined for mutations in the regulatory genes by nucleotide sequencing analysis. The PCR-amplicon size of the regulatory genes including *adeR*, *adeS*, *adeL* and *adeN* was 897 bp, 1,236 bp, 1,282 bp and 753 bp, respectively (Figure 12).

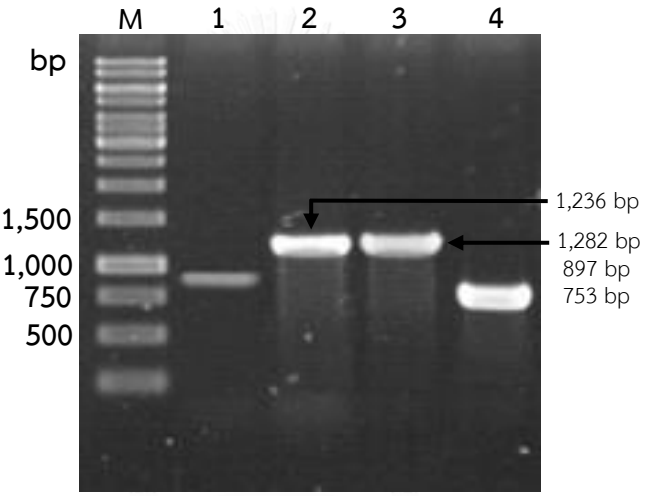

Figure 12: PCR amplicons of regulatory genes. Lane M, 1 kb DNA marker; Lane 1-4, the result of the PCR reaction amplifying *adeR*, *adeS*, *adeL* and *adeN*

4.6.1 The *A. baumannii* clinical isolates from humans (n=10)

Ten *A. baumannii* clinical isolates from humans were examined for the presence of mutations in the regulatory genes including *adeR*, *adeS*, *adeL* and *adeN* (Table 11). The main findings of the mutations in the RND multidrug efflux pump regulatory genes in the human isolates were as follows:

Mutation of AdeR was found in six human isolates expressing *adeB* (i.e. ABJ058, ABJ111, ABJ121 and ABJ203). Non-*adeB* expressing isolates including ABJ061 and ABJ099 also carried mutations in AdeR. The amino substitution Pro241-Leu was the most commonly found in AdeR and was present in all six isolates.

Five human isolates carried mutations in AdeS and had different mutational pattern. The amino substitution Ala153-Thr was most commonly found in AdeS, which was present in five of six human isolates. The four amino substitution Gly41-Ala, Trp42-Gly, Ile43-Leu and Ser44-Val followed by nonsense mutation Leu45-Ter were detected in ABJ178, with no expression of *adeB*. The presence of mutations in AdeR and/or AdeS did not result in overexpression of *adeB* in *A. baumannii* clinical isolates from humans (0.1 to 0.2 fold *adeB* expression in comparison to *A. baumannii* ATCC19606).

Three human isolates carried amino acid substitution in AdeL but was not identified in the others (six isolates). The mutations found were Arg2-Cys, Asp227-Ala and Gln264-Arg. The amino substitution Gln264-Arg was found in two human isolates including ABJ111 and ABJ121. No expression of *adeG* was detected in the isolates with the AdeL mutation.

All human isolates, except ABJ162 harbored AdeN amino acid change. The mutations found were Met174-Thr, Met197-Thr and Gly177-Ter. The amino substitution Met197-Thr was commonly found in AdeN that was detected in eight of nine human isolates. The nonsense mutation Gly177-Ter was found in ABJ111 with no

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overexpression of *adeJ*. No mutation was found in AdeN in ABJ162 that overexpressed *adeJ* (5 fold).

4.6.2 The *A. baumannii* clinical isolates from animals (n=15)

Fifteen *A. baumannii* animal clinical isolates were examined for the presence of mutations in AdeL and AdeN genes (Table 12). The main findings of the mutations in the RND multidrug efflux pump regulatory genes in the animal isolates were as follows:

The amino substitution Ile145-Val in AdeL was found in ABJ302 with the highest expression level of *adeG* (108.4 fold). No amino acid changes of whole gene sequence of *adeL* were observed in the other animal isolates.

Ten animal isolates contained the mutations of AdeN, while the other two isolates (ABJ303 and ABJ316) did not carry any mutations. Despite several attempts, the PCR amplicon of *adeN* was not obtained from ABJ323, ABJ317 and ABJ325. The amino substitution Met197-Thr was most commonly found in AdeN in the animal isolates. A duplication of amino acid Val44 (Val44dup) in AdeN was found in two animal isolates (ABJ329 and ABJ321), of which both overexpressed *adeJ* (7.8 and 16.7 fold respectively).

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4.7 Contribution of multidrug efflux systems in biocide-antibiotic cross-resistance

4.7.1 Spontaneous mutants after exposure to the biocides

4.7.1.1 The *A. baumannii* clinical isolates from humans

No spontaneous mutant was obtained after exposure to benzalkonium chloride and chlorhexidine. Triclosan-resistant mutants including ABJ114-1, ABJ159-1, ABJ174-1, ABJ178-1 and ABJ208-1 were obtained from five selected human isolates (ABJ114, ABJ159, ABJ174, ABJ178 and ABJ208), respectively. One colony from each triclosan-resistant mutant strain was collected and used for further study. These triclosan-resistant derivatives grew in LB broth supplemented with triclosan at the concentration above 4-fold of MIC.

4.7.1.2 The *A. baumannii* clinical isolates from animals

No spontaneous mutant was obtained after exposure to benzalkonium chloride and chlorhexidine. Triclosan-resistant mutants including ABJ302-1, ABJ303-1, ABJ311-1, ABJ316-1, ABJ317-1, ABJ319-1, ABJ320-1, ABJ321-1, ABJ323-1, ABJ324-1, ABJ329-1 and ABJ331-1 were obtained from 12 selected animal isolates (ABJ302, ABJ303, ABJ311, ABJ316, ABJ317, ABJ319, ABJ320, ABJ321, ABJ323, ABJ324, ABJ329 and ABJ331), respectively. One colony from each triclosan-resistant mutant strain was collected and used for further study.

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4.7.2 Biocide and antimicrobial susceptibility of triclosan-resistant mutants

4.7.2.1 Triclosan-resistant mutants of the *A. baumannii* clinical isolates from humans

The susceptibilities of triclosan and other antimicrobial agents of the *A. baumannii* clinical isolates from humans and their triclosan-resistant mutant derivatives are shown in table 13.

Cross-resistance between triclosan and antimicrobial agents was observed in four from five triclosan-resistant mutants (i.e. ABJ114-1, ABJ159-1, ABJ174-1 and ABJ178-1), of which MIC of triclosan increased 16 to 128 fold in comparison to their parental strains.

The significant increase (4-fold higher than that in the isogenic parental strain) of amikacin MIC was observed in ABJ159-1 and ABJ174-1. For ABJ114-1 and ABJ174-1, a 4-fold increase of the MIC for ceftazidime and streptomycin was also observed. The increase of the MIC for carbenicillin and piperacillin in ABJ114-1, ABJ159-1, ABJ174-1 and ABJ178-1 was up to 32 fold compared to their parental strains. A 4-fold increase of MIC for ciprofloxacin was found in three triclosan-resistant mutants of ABJ114, ABJ159 and ABJ174 i.e. ABJ114-1, ABJ159-1 and ABJ174-1, respectively. The significant increase of the MIC for tetracycline was observed only in ABJ114-1. No significant increase of MIC for aztreonam, chloramphenicol, erythromycin, gentamicin, kanamycin, neomycin, spectinomycin and trimethoprim was observed in all triclosan-resistant mutants.

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Table 13: Susceptibility of triclosan and other antimicrobial agents among the triclosan-resistant mutants and their parental strains of the *A. baumannii* clinical isolates from humans

Antimicrobials/ biocides	MIC (μg/ml)[a]									
	ABJ114	ABJ114-1*	ABJ159	ABJ159-1*	ABJ174	ABJ174-1*	ABJ178	ABJ178-1*	ABJ208	ABJ208-1*
triclosan	0.5	64	0.5	8	0.5	16	1	64	1	64
amikacin	8	16	8	32	8	32	8	8	8	16
aztreonam	>256	>256	>256	>256	32	32	64	64	128	128
carbenicillin	32	256	32	1024	32	512	32	512	>2048	>2048
ceftazidime	16	64	16	16	16	64	16	32	256	256
chloramphenicol	128	128	128	128	128	128	128	128	256	256
ciprofloxacin	0.25	1	0.25	1	0.25	1	1	1	32	64
erythromycin	64	64	>128	>128	>128	>128	>128	>128	>128	>128
gentamicin	2	2	2	4	2	2	2	2	2	2
kanamycin	8	8	8	16	8	8	8	8	8	8
neomycin	8	8	8	8	8	8	8	8	8	16
piperacillin	32	1024	32	>1024	32	>1024	32	>1024	>1024	>1024
spectinomycin	1024	1024	64	64	>1024	>1024	>1024	>1024	64	64
streptomycin	32	128	16	32	32	128	32	32	>1024	>1024
tetracycline	0.25	16	8	8	16	16	16	16	128	128
trimethoprim	>128	>128	128	128	32	32	32	32	64	64

[a], underlined values indicate MIC that are increased by at least 4-fold in the triclosan-resistant mutants compared to their parental strains.

*, isogenic triclosan-resistant mutant derivatives of the corresponding strains.

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4.7.2.2 Triclosan-resistant mutants of the *A. baumannii* clinical isolates from animals

The susceptibilities of triclosan and other antimicrobial agents of the triclosan-resistant mutant derivatives and their parental strains of the *A. baumannii* clinical isolates from animals are shown in table 14.

A ≥4-fold increase of the triclosan MIC was found in all animal triclosan-resistant mutant strains (n=12). A 4 to 16 fold increase of the MIC for aztreonam was observed in nine triclosan-resistant mutants i.e. ABJ302-1, ABJ316-1, ABJ317-1, ABJ319-1, ABJ320-1, ABJ321-1, ABJ324-1, ABJ329-1 and ABJ331-1. The MIC of erythromycin increased 4 to 256 fold in eight triclosan-resistant mutants including ABJ302-1, ABJ303-1, ABJ316-1, ABJ320-1, ABJ321-1, ABJ323-1, ABJ324-1 and ABJ331-1. A ≥4-fold increase of the MIC for carbenicillin and ciprofloxacin was found in six and four triclosan-resistant mutants, respectively.

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A ≥4-fold increase of the MIC for 10 antimicrobial drugs was observed in ABJ302-1. For all triclosan-resistant mutant derivatives of the animal isolates, the MIC value of amikacin, chloramphenicol, gentamicin, kanamycin and streptomycin was not changed.

Table 14: Susceptibility of triclosan and other antimicrobial agents among the triclosan-resistant mutants and their parental strains of the *A. baumannii* clinical isolates from animals

Antimicrobials/ biocides	MIC (μg/ml)[a]																							
	ABJ302	ABJ302-1*	ABJ303	ABJ303-1*	ABJ311	ABJ311-1*	ABJ316	ABJ316-1*	ABJ317	ABJ317-1*	ABJ319	ABJ319-1*	ABJ320	ABJ320-1*	ABJ321	ABJ321-1*	ABJ323	ABJ323-1*	ABJ324	ABJ324-1*	ABJ329	ABJ329-1*	ABJ331	ABJ331-1*
triclosan	1	16	4	32	2	64	2	32	0.5	32	8	64	1	256	2	256	0.5	32	1	64	1	32	1	>256
amikacin	1	2	8	8	8	8	2	2	2	2	8	8	8	16	2	2	64	64	2	2	2	2	4	8
aztreonam	8	128	16	32	32	32	32	256	32	256	16	256	32	128	32	256	>256	>256	32	256	32	128	32	256
carbenicillin	8	128	16	64	16	32	16	1024	16	128	>1024	>1024	64	64	1024	1024	>1024	>1024	32	512	32	32	128	512
ceftazidime	0.5	16	4	16	8	16	4	32	4	32	8	256	8	32	16	32	>256	>256	8	128	16	32	8	128
chloramphenicol	128	128	128	128	128	128	256	256	256	256	>256	>256	256	256	>256	>256	32	32	256	256	>256	>256	256	256
ciprofloxacin	0.03	1	32	64	64	64	16	>64	0.06	0.25	64	64	64	64	64	64	1	32	64	64	32	64	>64	>64
erythromycin	0.5	128	16	64	32	64	16	64	16	16	>128	>128	32	128	32	128	16	64	32	128	>128	>128	16	128
gentamicin	0.5	1	16	16	8	16	1	1	2	2	8	8	8	8	>128	>128	8	8	2	2	2	2	8	8
kanamycin	2	4	4	8	4	4	1	1	1	1	4	4	4	8	32	32	512	512	2	2	2	2	4	4
neomycin	1	4	8	8	8	8	8	8	8	8	8	8	8	8	8	8	64	64	16	16	8	8	8	8
piperacillin	4	64	2	32	16	32	2	256	8	64	16	512	16	64	64	256	>512	>512	16	256	32	32	>512	>512
spectinomycin	32	128	1024	1024	512	512	128	128	64	64	1024	1024	512	1024	64	64	>1024	>1024	256	256	128	128	256	256
streptomycin	4	4	128	128	1024	1024	1024	1024	4	4	>1024	>1024	1024	1024	16	16	16	16	256	256	32	32	512	512
tetracycline	0.5	2	128	128	>256	>256	>256	>256	1	2	>256	>256	>256	>256	>256	>256	0.5	0.5	>256	>256	>256	>256	>256	>256
trimethoprim	1	16	4	8	64	64	32	32	32	32	64	64	64	64	64	64	>128	>128	64	64	64	64	64	64

[a], underlined values indicate MIC that are increased by at least 4-fold in the triclosan-resistant mutants compared to their parental strains.

*, isogenic triclosan-resistant mutant derivatives of the corresponding strains.

4.7.3 Distribution of RND, MFS and MATE multidrug efflux systems in triclosan-resistant mutants

The PCR amplicon size of RND efflux transporters including *adeB*, *adeG* and *adeJ* were 153 bp, 245 bp and 346 bp respectively. The PCR amplicon size of *amvA* (MFS transporter) and *abeM* (MATE transporter) was 1,451 bp and 1,330 bp, respectively as shown in figure 13.

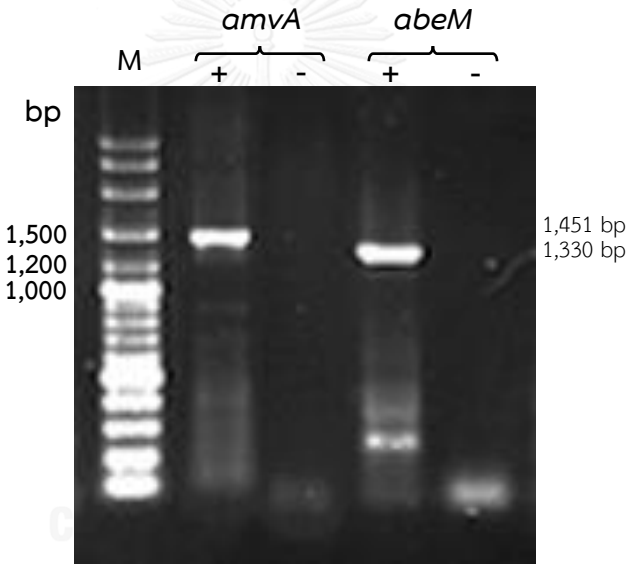

Figure 13: RT-PCR result of *amvA* and *abeM*. Lane M, 100 bp DNA marker; +, with reverse transcriptase and -, without reverse transcriptase

4.7.3.1 The *A. baumannii* human clinical isolates: triclosan-resistant mutants and their parental strains

The expression of the transporters of the RND, MFS and MATE efflux systems in triclosan-resistant mutants and their parental strains originated from humans is shown in table 15.

The expression of five efflux pump genes including *adeB*, *adeG*, *adeJ*, *amvA* and *abeM* was determined in ABJ174 and its triclosan-resistant mutant derivative ABJ174-1. The expression of *adeB*, *adeJ* and *abeM* was detected in ABJ114 and ABJ114-1. The expression of *adeB*, *adeG*, *adeJ* and *abeM* was positive in ABJ159. However, the expression of *adeG* was negative in ABJ159-1. All human isolates both the parental strains and theirs triclosan-resistant mutant derivatives were positive for *adeJ* and *abeM* expression. The expression of *adeG* was positive in ABJ208-1, but negative in ABJ208. ABJ178 expressed *adeG* but not *adeB*. In contrast, its triclosan-resistant mutant derivative ABJ178-1 expressed *adeB* not *adeG*.

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Table 15: Distribution of multidrug efflux system expression among triclosan-resistant mutants and their parental strains of the *A. baumannii* clinical isolates from humans and animals

		RND family[a]			MFS[a]	MATE family[a]
	Strains*	AdeB	AdeG	AdeJ	AmvA	AbeM
Human clinical isolates	ABJ114	+	-	+	-	+
	ABJ114-1	+	-	+	-	+
	ABJ159	+	+	+	-	+
	ABJ159-1	+	-	+	-	+
	ABJ174	+	+	+	+	+
	ABJ174-1	+	+	+	+	+
	ABJ178	-	+	+	+	+
	ABJ178-1	+	-	+	+	+
	ABJ208	-	-	+	+	+
	ABJ208-1	-	+	+	+	+
Animal clinical isolates	ABJ302	-	+	+	+	+
	ABJ302-1	-	+	+	+	+
	ABJ303	+	+	+	+	+
	ABJ303-1	+	-	+	+	+
	ABJ311	+	+	+	-	-
	ABJ311-1	+	+	+	-	+
	ABJ316	+	+	+	-	-
	ABJ316-1	-	-	+	-	-
	ABJ317	-	+	+	-	-
	ABJ317-1	-	-	+	-	-
	ABJ319	+	+	+	+	+
	ABJ319-1	+	+	+	+	+
	ABJ320	+	+	+	-	+
	ABJ320-1	+	+	+	-	+
	ABJ321	+	+	+	+	+
	ABJ321-1	-	+	+	+	+
	ABJ323	-	-	+	-	-
	ABJ323-1	-	+	-	-	-
	ABJ324	-	-	+	-	-
	ABJ324-1	+	-	+	-	+
	ABJ329	-	+	+	+	+
	ABJ329-1	-	+	+	+	+
	ABJ331	+	+	+	-	-
	ABJ331-1	+	+	+	-	+

[a] + and -, denote the presence or absence of the relevant genes by RT-PCR, respectively.

*, the isolates with "-1" are the isogenic triclosan-resistant mutant derivatives of the corresponding strains.

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4.7.3.2 The *A. baumannii* animal clinical isolates: triclosan-resistant mutants and their parental strains

The expression of the RND, MFS and MATE efflux transporter in triclosan-resistant mutants and their parental strains originated from animals is shown in table 15. The main findings of the distribution of the multidrug efflux systems in triclosan-resistant mutants and their parental strains were as follows:

The same expression pattern of multidrug efflux systems was observed in ABJ302, ABJ319, ABJ320 and ABJ329 with their triclosan-resistant mutants. The expression of *adeG* was detected in ABJ303, ABJ316 and ABJ317, but not in all of their triclosan-resistant mutants. The expression of *adeB* was negative in ABJ316-1 and ABJ321-1, but positive in their parental strains i.e. ABJ316 and ABJ321. The expression of *adeJ* was not found in ABJ323-1. In the same time, the expression of *adeG* was changed from negative in ABJ323 to positive in ABJ323-1.

The expression of *abeM* was found in ABJ311-1, ABJ324-1 and ABJ331-1 but not in their isogenic parental strains that were ABJ311, ABJ324 and ABJ331.

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4.7.4 Expression level of RND multidrug efflux systems in triclosan-resistant mutants

4.7.4.1 The *A. baumannii* human clinical isolates: triclosan-resistant mutants and their parental strains

The expression level of RND efflux systems among the triclosan-resistant mutant derivatives and their parental strains of the *A. baumannii* human clinical isolates is shown in table 16.

No transcription of AdeB and AdeG was observed in all triclosan-resistant mutant derivatives of the human isolates compare to *A. baumannii* ATCC19606. The expression level of *adeJ* in the triclosan-resistant mutants varied from 0.1 to 4.7 fold. Overexpression of *adeJ* was found in ABJ174-1 (4.7 fold) while the expression level of *adeJ* in ABJ174 was 1.4 fold.

4.7.4.2 The *A. baumannii* animal clinical isolates: triclosan-resistant mutants and their parental strains

The expression level of RND efflux systems among the triclosan-resistant mutant derivatives and their parental strains of the *A. baumannii* animal clinical isolates is shown in table 16.

All the triclosan-resistant mutants of the animal isolates did not expressed *adeB*. The expression level of *adeG* in the triclosan-resistant mutants varied from 0.1 to 3.7 fold. No transcription of AdeG was observed in ABJ311-1, ABJ319-1, ABJ320-1

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and ABJ329-1 while their parental strains overexpressed *adeG*. The overexpression of *adeG* was found only in ABJ302-1 (3.7 fold).

The expression level of *adeJ* in the triclosan-resistant mutant strains varied from 0.2 to 3.3 fold. Overexpression of *adeJ* was found in four triclosan-resistant mutant derivatives of the animal isolates including ABJ302-1, ABJ320-1, ABJ321-1 and ABJ331-1.

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Table 16: Expression of the RND efflux systems and mutation in the corresponding regulatory regions among triclosan-resistant mutant derivatives and their parental strains of the *A. baumannii* clinical isolates from humans and animals

		Transcription level[a]			Regulatory mutation[b]	
	strains*	*adeB*	*adeG*	*adeJ*	AdeL	AdeN
Human	ABJ114	0.1	-	1.2	-	Met197-Thr
clinical	ABJ114-1	0.1	-	0.1	-	His9_Pro16del, Thr18_Leu28del
isolates	ABJ159	0.1	1.0	1.5	-	Met197-Thr
	ABJ159-1	0.1	-	0.3	-	Met197-Thr
	ABJ174	0.1	0.8	1.4	-	-
	ABJ174-1	0.1	0.9	4.7	-	-
	ABJ178	-	3.0	2.0	-	Met197-Thr
	ABJ178-1	0.1	-	0.3	-	Gln147-Pro, Met197-Thr
	ABJ208	-	-	1.1	-	Met174-Thr, Met197-Thr
	ABJ208-1	-	0.9	2.5	-	Met174-Thr, Met197-Thr
Animal	ABJ302	-	108.4	11.6	Ile145-Val	Asn195-His, Met197-Thr
clinical	ABJ302-1	-	3.7	3.0	-	Asn195-His, Met197-Thr
isolates	ABJ303	2.4	19.6	6.3	-	-
	ABJ303-1	0.1	-	1.6	-	Ala32-Thr
	ABJ311	1.3	13.4	13.3	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
	ABJ311-1	0.1	0.6	2.6	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
	ABJ316	0.2	2.0	2.0	-	-
	ABJ316-1	-	-	0.2	-	Gly69-TyrfsTer28
	ABJ317	-	0.1	8.0	-	ND
	ABJ317-1	-	-	2.9	-	ND
	ABJ319	2.4	7.4	14.1	-	Pro16-Ser, Met186-Ile, Met197-Thr
	ABJ319-1	0.1	0.8	2.7	-	Pro16-Ser, Met186-Ile, Met197-Thr
	ABJ320	2.2	33.4	14.0	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
	ABJ320-1	0.1	0.4	3.0	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
	ABJ321	0.1	62.3	16.7	-	Val44dup
	ABJ321-1	-	1.0	3.3	-	Val44dup
	ABJ323	-	-	0.1	-	ND
	ABJ323-1	-	0.1	-	-	ND
	ABJ324	-	-	0.1	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
	ABJ324-1	0.1	-	2.6	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
	ABJ329	-	19.8	7.8	-	Val44dup
	ABJ329-1	-	0.5	2.1	-	Val44dup
	ABJ331	2.2	10.3	2.1	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr
	ABJ331-1	0.1	2.4	3.3	-	Gly21-Ser, Gln94-Arg, Gln103-His, Met197-Thr

*, the isolates with "-1" are the isogenic triclosan-resistant mutant derivatives of the corresponding strains.

[a]-, no expression.

[b]-, no mutations found; ND, not detected.

4.7.5 Mutation in the regulatory genes in triclosan-resistant mutants

4.7.5.1 The *A. baumannii* human clinical isolates: triclosan-resistant mutants and their parental strains

The mutations of AdeL and AdeN among the triclosan-resistant mutant derivatives and their parental strains of the *A. baumannii* human clinical isolates are shown in table 16.

No mutation of AdeL was detected in triclosan-resistant mutants and their parental strains from humans. A deletion of 19 amino acids in AdeN was observed in ABJ114-1 with no transcription of AdeJ.

No mutation of AdeN was found in ABJ174 and ABJ174-1, of which the expression level of *adeJ* increased from 1.4 fold in ABJ174 to 4.7 fold in ABJ174-1. An amino acid substitution Gln147-Pro of AdeN was found in ABJ178-1, of that did not overexpressed *adeJ*.

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4.7.5.2 The *A. baumannii* animal clinical isolates: triclosan-resistant mutants and their parental strains

The mutations of AdeL and AdeN among the triclosan-resistant mutant derivatives and their parental strains of the *A. baumannii* animal clinical isolates are shown in table 16.

No mutation of AdeL was found in all the triclosan-resistant mutant strains of the animal isolates. The amino acid substitution Ile145-Val in AdeL was found in ABJ302 with high expression level of AdeG (108.4 fold). The mutation was absent and the

expression level decreased from 108.4 in ABJ302 to 3.7 fold in ABJ302-1. A frame shifts deletion with Gly69 replace by Tyr leading to a new reading frame ending at a stop at position 28 (counting starts with the Tyr as amino acid 1) in AdeN was found in ABJ316-1, of which no mutation was observed in ABJ316. However, overexpression of *adeJ* was not observed in both ABJ316 and ABJ316-1.

The amino acid substitution Ala32-Thr in AdeN was detected in ABJ303-1 without expression of *adeJ*. Vice versa, ABJ303 exhibited overexpression of *adeJ* with no mutation of AdeN.

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CHAPTER V

DISCUSSIONS

A. baumannii is one of the most problematic pathogens for the health care facilities because of its ability to develop resistance to multiple antimicrobial agents via multiple mechanisms (Fournier and Richet, 2006). The microorganism mainly affects ill patients in intensive care unit (ICU) and inflicts hospital acquired infection such as ventilator-associated pneumonia (VAP), wound infection and septicemia (Dijkshoorn et al., 2007). As seen in humans, *A. baumannii* infection in animals can cause life-threatening illness and has been involved in high morbidity and mortality rate (Francey et al., 2000; Brachelente et al., 2007). Up to date, multidrug resistant (MDR) *A. baumannii* has been reported increasingly and most of them are resistant to commonly used antimicrobial agents in human and veterinary medicine (Michalopoulos and Falagas, 2010). Besides antibiotics, biocides have been frequently used for prevention of bacterial spread in healthcare facilities. However, it is possible that bacteria may develop resistance to biocides and promote cross-resistance to other antimicrobial agents (Carey and McNamara, 2014; Webber et al., 2015). It is well documented that multidrug efflux pumps are the major mechanisms responsible for such antimicrobial cross-resistance (Chuanchuen et al., 2001; Coyne et al., 2011).

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In this study, we investigated the antimicrobial resistance phenotype and the expression of RND efflux systems in both *A. baumannii* clinical isolates from humans and animals. Cross-resistance between selected biocides and antibiotics and its effect on expression of the RND, MFS and MATE multidrug efflux pumps were examined.

5.1 Presence of *A. baumannii* clinical isolates from humans and animals

In general, identification of *Acinetobacter* species is complicate. The phenotypic identification method yielded high differentiation ability with high accuracy at genus level (Seifert et al., 1997), but cannot discriminate between *A. baumannii* and *Acinetobacter* genomic species 13TU (Gerner-Smidt et al., 1991). Species identification by the API 20NE and VITEX 2, that are the semi-automated commercial identification systems does not differentiate the strains in *A. calcoaceticus-baumannii* complex (*Acb* complex) and genomic species 13TU (Bernards et al., 1996). Therefore, several molecular techniques have been developed for identification of *Acinetobacter* species (Dolzani et al., 1995; Ehrenstein et al., 1996; Janssen et al., 1997). Among these, Amplified Ribosomal DNA Restriction Analysis (ARDRA) with five restriction enzyme (*Alu*I, *Mbo*I, *Cfo*I, *Rsa*I and *Msp*I) is one of the commonly used methods for identification of *Acinetobacter* species (Dijkshoorn et al., 1998; Peleg et al., 2008) and has been used as the confirmation method of the *baumannii* species (Ramirez et al., 2010; Kong et al., 2011). Therefore, the *A. baumannii* clinical isolates from humans and animals in this study were confirmed using ARDRA with restriction enzyme *Alu*I, *Mbo*I and *Cfo*I

that is sufficient to differentiate the *A. baumannii* from the *Acb* complex (Vaneechoutte et al., 1995; Dijkshoorn et al., 1998; Ramirez et al., 2010).

All the *Acinetobacter* spp. from the bacterial strain collection of Siraraj hospital (n=100) were originated from patients with different specimens (see appendix) and were identified as *A. baumannii* by ARDRA method. This is not far from our expectation since *A. baumannii* is frequently found in the hospitalized patients and has been commonly associated with hospital outbreaks (Tankovic et al., 1994; Maragakis and Perl, 2008).

In animals, *A. baumannii* has been reported as an emerging opportunistic pathogen in hospitalized domestic pets (Francey et al., 2000; Endimiani et al., 2011; Zordan et al., 2011). In this study, the percentage of *A. baumannii* isolates from animals was 14.29% and most animal isolates were obtained from companion animals including dogs, cats and rabbits. This may not be surprising because most animal samples were submitted to the Pathology unit from small animal hospitals. Previous studies reported the isolation of *A. baumannii* from animals (i.e. dogs, cats, horses, goats, ducks, pigeons, donkeys and chickens) with various prevalence ranging from 5.1 to 8.5% (Black et al., 2009; Kempf et al., 2012; Belmonte et al., 2014; Pailhories et al., 2015). The approximately two time higher percentage found in this study may be related to the sample collection criteria that mainly focused on animals with respiratory infection and chronic diseases. This agreed with the high detection rate of *A. baumannii* in patients with respiratory infection and chronic diseases (Ntusi et al.,

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2012). During the last decade, *A. baumannii* has been also found in food animals i.e. pigs and cattle (Hamouda et al., 2011; Zhang et al., 2013). However, the prevalence of *A. baumannii* in livestock (1.2%) was not as high as that in companion animals (8.5%) (Hamouda et al., 2011; Pailhories et al., 2015). This could be partly because *A. baumannii* is not included in routine bacterial examination in livestock and mass medication has been applied for treatment in sick animals before a cause of infection was defined. In this study, one pig sample was positive to *A. baumannii* confirming that *A. baumannii* could also be present in livestock. From these observations, *A. baumannii* was not disseminated only in companion animals but also distributed in livestock.

5.2 Antimicrobial susceptibility of *A. baumannii* clinical isolates from humans and animals

One of the main findings of this study was the high percentage of MDR *A. baumannii* from both humans (98%) and animals (70%). A variety definition has been proposed for MDR *A. baumannii*. MDR *A. baumannii* was previously described based on the different protocol of *A. baumannii* treatment and the availability of antimicrobial agents in the regions (Michalopoulos and Falagas, 2010). MDR *A. baumannii* was defined as the isolates resistant to all drugs including piperacillin/tazobactam, defepime, ceftazidime, aztreonam, ciprofloxacin, gentamicin and tobramycin by Abbo et al. (2005). Later, MDR *A. baumannii* was defined by Magiorakos et al. (2012) as those being resistant to at least one drug in at least three antimicrobial categories including

aminoglycosides, antipseudomonal carbapenems, antipseudomonal fluoroquinolones, antipseudomonal penicillins + ß-lactamase inhibitors, extended-spectrum cephalosporins, folate pathway inhibitors, penicillin + ß-lactamase inhibitors, polymyxins and tetracyclines. Likewise, Ghajavand et al. (2015) defined MDR *A. baumannii* as the strains that were resistant to at least three from five difference classes of antimicrobial agents including cephalosporins, carbapenems, ampicillin-sulbactam, fluoroquinolones and aminoglycosides. In this study, we defined MDR *A. baumannii* as the isolates resistant to at least six antimicrobial agents (Gu et al., 2007; Poonsuk et al., 2012). This definition is compatible with various *A. baumannii* treatment protocols and independent from the antimicrobials availability.

MDR *A. baumannii* is recognized as one of the most difficult Gram-negative pathogens for treatment and has been increasingly reported worldwide (Maragakis and Perl, 2008). The percentage of MDR *A. baumannii* among the human clinical isolates in this study was very high (98%), in agreement with previous reports in Pakistan and Iran (100%) (Begum et al., 2013; Ghajavand et al., 2015), Sudan (97%) (Omer et al., 2015) and Algeria (94%) (Khorsi et al., 2015). However, the resistance rate in this study was higher than those reported in some other regions such as North America (63% in The US) (CDC, 2013), Europe (54% in Italy, 6% in German) (Wadl et al., 2010; De Francesco et al., 2013) and Asia (51% in Malaysia, 72% in Taiwan) (Lin et al., 2013; Janahiraman et al., 2015). When compared to previous study in Thailand, the percentage of MDR *A. baumannii* found in the present study was higher (Inchai et al.,

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2015). The resistance rate of amikacin (78%), aztreonam (95%), gentamicin (89%), tetracycline (93%) in this study was high and consistent with previous report from SENTRY antimicrobial program in the Western Pacific regions (Yau et al., 2009). In contrast, resistance rate of ceftazidime, ciprofloxacin and piperacillin from this study was lower (93% vs 100%, 81% vs 97% and 91% vs 100%, respectively) when compared with the report from the same program (Yau et al., 2009). The high resistance rate (99%) of chloramphenicol in this study was compatible to the report in China (97%) (Gu et al., 2007). Furthermore, kanamycin resistance rate found in this study (87%) was similar to that reported in Algeria (86%) (Bakour et al., 2013). Taken together, these results support that MDR *A. baumannii* spreads worldwide.

Among the animal isolates, the percentage of MDR *A. baumannii* clinical isolates was high (70%) but still lower than that in the human isolates (98%). Resistance rates of the animal isolates for all the antimicrobial agents tested in this study were lower than those of the human isolates except in chloramphenicol. MDR *A. baumannii* in animals was first discovered in horse (Vaneechoutte et al., 2000). Later, MDR *A. baumannii* was isolated from both companion animals i.e. dogs, cats, horses (Boerlin et al., 2001; Brachelente et al., 2007; Black et al., 2009; Zordan et al., 2011; Pomba et al., 2014; Herivaux et al., 2016) and livestock i.e. cattle, pigs and fowls (Zhang et al., 2013; Al Bayssari et al., 2015). The MDR *A. baumannii* isolates have been most commonly detected in companion animals. It was suggested that such high prevalence was associated with the close contact between owners and their pets, resulting in higher

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chance of organism transmission among them (Muller et al., 2014). Still, we cannot make a conclusion for the observation in this study because of the sampling bias. Most animal samples were collected from companion animals.

Previous studies demonstrated that efflux pump inhibitors (EPI) could partially reverse antimicrobial resistance in *A. baumannii* (Pannek et al., 2006; Yang and Chua, 2013). Reserpine has been known to be a competitive inhibitor that mainly inhibit non-RND family efflux pumps for a quite long time (Akiyama et al., 1988). At the same time, carbonyl cyanide *m*-chlorophenylhydrozone (CCCP) has been known as an energy uncoupler that interferes the mechanisms using Proton Motive Force (PMF) as energy, including RND efflux pump (Pages et al., 2005). Few studies showed that addition of reserpine or CCCP could reduce resistance rate of *A. baumannii* on fluoroquinolones (Shi et al., 2005; Lin et al., 2009). In the present study, the presence of reserpine had no effect on resistance rate of ciprofloxacin in both *A. baumannii* isolates from humans and animals. The presence of CCCP also had no effect on ciprofloxacin resistance rate in the animal isolates, but decreased the rate by 3% in the human isolates. This result was compatible with previous study that revealed ciprofloxacin resistance rate in *A. baumannii* was not affected by reserpine or CCCP (Park et al., 2011; Kuo et al., 2012). This suggests that the responsible mechanisms for ciprofloxacin resistance are likely not efflux pump. In this study, the addition of CCCP resulted in a ≥4-fold reduction of tetracycline MIC, in agreement with a previous study (Beheshti et al., 2014). We found that the addition of reserpine was more effective than CCCP in reduction of the

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resistance rates of aztreonam and tetracycline. This data could imply that PMF-dependent efflux was not a major resistance mechanisms responsible for aztreonam and tetracycline resistance in *A. baumannii* clinical isolates in this collection. In the exception of aztreonam and tetracycline, the addition of CCCP was more potent than reserpine in enhancing the antimicrobial activity and regaining antimicrobial susceptibility among *A. baumannii* clinical isolates from humans and animals. These findings suggested that PMF-dependent efflux including RND efflux pumps contribute to multidrug resistance phenotype among *A. baumannii* in this collection (Nikasa et al., 2013).

5.3 Contribution of multidrug efflux systems in *A. baumannii* clinical isolates from humans and animals

AdeB was suggested as an efflux pump specific to *Acinetobacter* genomic species 2 or *A. baumannii* (Chu et al., 2006). This is in agreement with this study where the presence of *adeB* was 83% in human isolates and 53% in animal isolates. Our results were consistent with a previous study, which reported that 84% of their 112 isolates carried *adeB* (Lin et al., 2009). However, our results were different from a previous study reporting that *adeB* was found in 70% of *A. baumannii* isolates (n=56) (Chu et al., 2006). AdeJ was observed in all *A. baumannii* clinical isolates in this study except three human isolates. This finding was similar to previous studies, suggesting

that *adeIJK* is responsible for intrinsic resistance to antimicrobial agents in *A. baumannii* (Damier-Piolle et al., 2008; Yoon et al., 2013).

In the present study, the expression pattern of AdeB-AdeJ (with/without AdeG) and AdeJ only was found in the human isolates (83% and 5%, respectively). This agreed with a previous study showing AdeB-AdeJ (83.9%) and AdeJ (6.3%) at the similar rate, respectively (Lin et al., 2009). These observations supported the notion that co-presence of AdeABC and AdeIJK efflux pumps could play an important role in antimicrobial resistance in *A. baumannii* (Magnet et al., 2001; Damier-Piolle et al., 2008).

AdeDE was first identified in *Acinetobacter* genomic species 3 only (Chau et al., 2004). Later, it was detected in genomic species 13TU and 17 (Chu et al., 2006). The presence of this efflux pump was shown to be species specific and did not coexist with AdeABC or AdeIJK (Lin et al., 2009). It is in agreement with the current study where no *adeE* expression was observed in all *A. baumannii* clinical isolates from humans and animals. This also suggested that the expression of AdeE could be used as indicator for differentiating *Acinetobacter* species (Lin et al., 2009; Luo et al., 2011).

Previous studies reported that mutations in conserved domains of AdeRS (Asp20-Asn, Ala91-Val and Pro116-Leu in AdeR / Ala94-Val, Gly103-Asp and Thr153-Met in AdeS) were associated with overexpression of AdeABC (Hornsey et al., 2010; Yoon et al., 2013). In AdeR, the amino acid substitutions Asp20-Asn in the phosphorylation site (Higgins et al., 2007), Ala91-Val and Pro116-Leu in the signal receiver domain of helix α5 (Hornsey et al., 2011) resulted in overexpression of AdeABC. In AdeS, the

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amino acid substitutions Gly103-Asp located between the sensor and the DHp domains (Hornsey et al., 2011) and Gly30-Asp (Coyne et al., 2010a) was shown to be responsible for overexpression of AdeABC. However, none of these amino acid substitutions were found in this study. In the present study, an Ala136-Val amino acid substitution in AdeR and a Gly186-Val amino acid substitution in AdeS were identified in the human isolates with no transcription of *adeB*. These two mutations were previously reported as a polymorphisms characteristics in clonal complexes (Yoon et al., 2013). It suggested that these mutations were not responsible for overexpression of AdeABC in *A. baumannii* clinical isolates. However, some studies revealed that mutations of AdeRS did not correlate with AdeABC expression (Bratu et al., 2008) and not all of AdeRS mutations were associated with overexpression of AdeABC (Chang et al., 2016). Therefore, further study e.g. the site-directed mutagenesis should be conducted to prove the effect of mutations on the transcription of AdeABC.

Whole sequence of *adeL*, a LysR-type transcriptional regulator family regulator of AdeFGH was analyzed. In ABJ302, an amino acid substitution Ile145-Val in AdeL was found together with 108.4-fold expression of *adeG*. The nonsense mutation Gln326-Ter and the amino acid substitution Val139-Lys in the putative recognition domain of AdeL resulting in overexpression of AdeG was previously reported (Coyne et al., 2010b). In contrast, we did not find any mutations in AdeL in ABJ328 overexpressing 214.8 fold AdeG. An amino acid substitution Gln256-Arg in AdeL that was not related to overexpression of *adeG* in MDR *A. baumannii* strains was previously reported (Yoon et

al., 2013). From these observations, it suggests the existence of other regulatory mechanisms responsible for expression of AdeFGH in addition to AdeL (Yoon et al., 2013).

The expression of AdeIJK has been shown to be regulated by AdeN, a Tet-R type regulator (Rosenfeld et al., 2012). Many different mutations of AdeN with various expression level of *adeJ* were observed in this study. In two isolates, ABJ321 and ABJ329, a duplication of Valine at position 44 (Val44dup) was found with overexpression of *adeJ* (16.7 and 7.8-fold respectively). The overexpression of *adeJ* associated with mutations of AdeN was previously demonstrated. A previous study showed that an amino acid substitution Asn194-Met led to a premature stop codon at position 211 in AdeN resulted in overexpression of *adeJ* (5-fold) (Rosenfeld et al., 2012). Another study demonstrated a 73 bp deletion at position 224 that introduced the loss of six predicted α-helices in AdeN and responsible for a 3.5-fold increase in *adeJ* expression (Fernando et al., 2014). However, the mutations mentioned above were not found in our strains in this study. The amino acid substitution Pro16-Ser in AdeN was found only in an *adeJ* overexpressing strain i.e. ABJ316. This mutation was previously found in both non-overexpressing and overexpressing *adeJ* isolates (Rumbo et al., 2013). It was suggested that Pro16-Ser was not associated with overexpression of AdeJ. It is possible that transcription levels of AdeJ may be independent from the mutations of AdeN among these *A. baumannii* clinical isolates. Therefore, the existence of

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additional regulatory mechanisms on *adeJ* expression other than mutations in regulatory gene, *adeN*, is suggested.

5.4 Cross-resistance between triclosan and antimicrobial agents in *A. baumannii* clinical isolates from humans and animals

Biocide-antibiotic cross-resistance mediated by multidrug efflux pumps has been previously reported in several Gram-negative bacterial species (Chuanchuen et al., 2001; Sanchez et al., 2005; Pagedar et al., 2011) including *A. baumannii* (Fernando et al., 2014). Previous study revealed that MIC value of biocides was correlated with the MIC value of amikacin, ceftazidime and ciprofloxacin in *Acinetobacter* spp. (Kawamura-Sato et al., 2010). In this study, 17 isolates of triclosan-resistant mutants were obtained from total 25 isolates by the *in vitro* exposure experiment. We observed a ≥4-fold increase in MIC for ciprofloxacin and aztreonam in seven and nine out of 17 triclosan-resistant mutants respectively. These results agreed with a previous study showing that a 2 fold increase in MIC for both antimicrobial drugs was found in the triclosan-resistant mutant (Fernando et al., 2014). A previous study demonstrated that all the triclosan-resistant isolates were also resistant to amikacin and tetracycline (Chen et al., 2009).

AbeM is a member of the MATE family multidrug efflux pump that effluxes several antimicrobial agents including triclosan, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, kanamycin, norfloxacin, ofloxacin and trimethoprim (Su et

al., 2005). Three triclosan-resistant mutant strains (ABJ311-1, ABJ324-1 and ABJ331-1) expressed AbeM, while their isogenic parental strains (ABJ311, ABJ324 and ABJ331) did not. This finding suggested that *abeM* was likely involved in the acquired resistance of triclosan in *A. baumannii* clinical isolates in this study. In contrast, previous studies showed that the expression of *abeM* was not contribute to triclosan-resistant in the *A. baumannii* human clinical isolates and triclosan-resistant mutant derivative of ATCC strain (Chen et al., 2009; Fernando et al., 2014). Therefore, further study is required to explore the exact contribution of *abeM* in triclosan and antibiotics resistance, for example, allele gene replacement.

AdeABC and AdeIJK, have been shown to mediate the reduced susceptibility to biocides in *A. baumannii* (Rajamohan et al., 2010b). In the present study, transcription of *adeB* was conversed from negative in the parental strains ABJ178 and ABJ324 to positive in the triclosan-resistant mutant derivatives ABJ178-1 and ABJ324-1. In contrast, *adeB* expression was changed from positive in ABJ316 and ABJ321 to negative in ABJ316-1 and ABJ321-1, respectively. Moreover, the transcription of *adeG*, which changed from positive in ABJ159, ABJ178 and ABJ316 to negative in ABJ159-1, ABJ178-1 and ABJ316-1 were also observed in this study. Among these triclosan-resistant mutant strains, a ≥4-fold increase in MIC for aztreonam, carbenicillin, ceftazidime, ciprofloxacin, erythromycin and piperacillin was also found. The transcription of *adeB* or *adeG* that conversed from positive to negative is expected to increase susceptibility to their antimicrobial substrates. It is not the case in this study.

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These observations suggested that the transcription of RND efflux pumps is not the sole mechanisms responsible for acquired resistance of triclosan in *A. baumannii* clinical isolates.

A decrease of transcription level of *adeG* was found in ABJ302-1, ABJ311-1, ABJ319-1, ABJ320-1, ABJ321-1, ABJ329-1 and ABJ331-1 compare to their isogenic parental strains. In ABJ302, 108.4 fold expression of *adeG* was found together with the amino acid substitution Ile145-Val in AdeL, but in ABJ302-1 the expression of *adeG* was decreased to 3.7 fold with no mutation in AdeL. In contrast, in the others isolates the expression of *adeG* was decreased from 7.4-62.3 fold in the parental strains to 0.4-2.4 fold in the triclosan-resistant mutants, while no mutation of AdeL was found in both triclosan-resistant mutants and their isogenic parental strains of these isolates. In addition, no alterations of AdeN were observed in ABJ174-1 and ABJ331-1 in comparison to ABJ174 and ABJ331, of which transcription of *adeJ* was raised from less than 3 fold in the parental strains to overexpression in the triclosan-resistant mutant strains. These results suggested that the expression of *adeG* or *adeJ* is not always associated with mutation of corresponding regulatory gene. Therefore, other mechanisms that were not included in this study such as mutations of *fabI* gene, which conferred increasing resistance for triclosan that well recognized in *E. coli* (McMurry et al., 1998) may be responsible for acquired resistance of triclosan in these *A. baumannii* clinical isolates. It confirms that exposure to triclosan can result in cross-resistance to other antimicrobial agents in *A. baumannii* clinical isolates from humans and animals.

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CONCLUSIONS AND SUGGESTIONS

From the observation in this study, we concluded that the wide spread of multidrug resistance among *A. baumannii* clinical isolates from humans and animals. The wide distribution of RND efflux systems among the human and animal clinical isolates was also demonstrated in the study. Transcription levels of AdeABC, AdeFGH and AdeIJK are independent on mutations of the recognized regulatory genes. The regulation of *adeABC*, *adeFGH*, *adeIJK* was complicated and may be involved with other regulatory mechanisms.

Our study highlight that more than 70% of *A. baumannii* clinical isolates from humans and animals are MDR *A. baumannii*. The antimicrobial resistance phenotype, which resistant to all 15 antimicrobial drugs tested is present among the human and animal isolates. Besides, the RND efflux expression pattern AdeB-AdeG-AdeJ positive is the predominant expression pattern in both the human and animal isolates. Therefore, these data suggested that the expression of RND efflux systems play an important role in the dissemination of multidrug resistance phenotype among the *A. baumannii* clinical isolates from humans and animals (Figure 14). However, this study was not designed for determine the circulation and transmission of *A. baumannii* between humans and animals.

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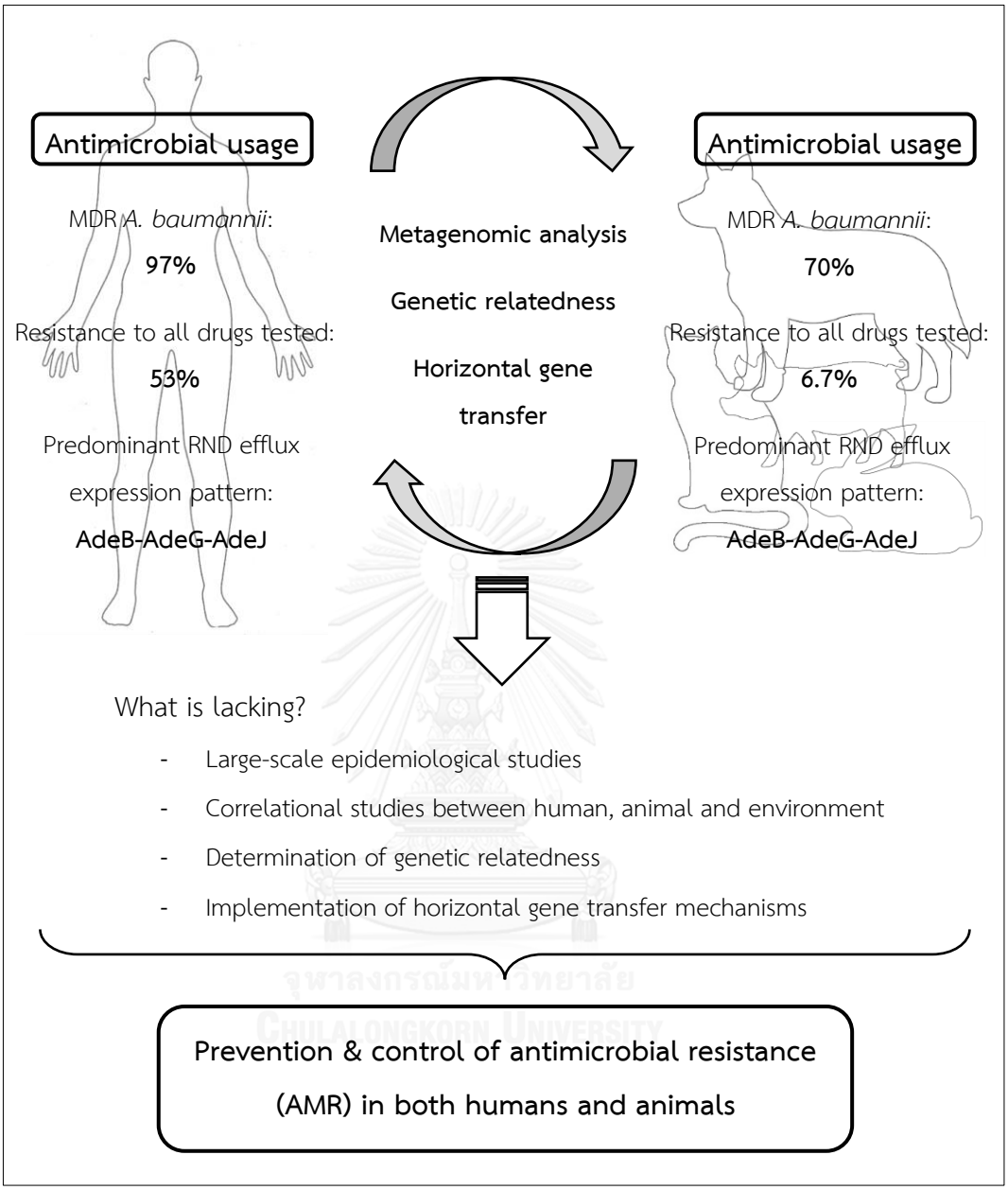

Figure 14: Dissemination of antimicrobial resistance phenotype and RND efflux expression pattern among *A. baumannii* human and animal clinical isolates, conclusions and suggestions of this study.

In addition, exposure of a clinically important biocides such as triclosan can cross-resistance to antimicrobial agents result in decrease antimicrobial susceptibility and leading to multidrug resistance in *A. baumannii* clinical isolates. However, our results showed that AdeABC, AdeFGH and AdeIJK are not a primary cause of the event and *A. baumnnnii* possesses multiple triclosan-resistant mechanisms.

The results from the present study affirmed that the appropriate use of antimicrobial agents and biocides in both human and veterinary medicine need to be addressed at regional and international level. Guideline for the prudent use of antimicrobials and biocides in human and veterinary medicine should be advocated. The development and implementation of control and prevention of antimicrobial resistance bacteria such as active surveillance of antimicrobial resistance in *A. baumannii* and other pathogenic bacteria, strengthen the public education should be encouraged.

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Additional suggestions and further studies are as follows:

- Large population sampling is required for large-scale epidemiological studies of *A. baumannii*.
- The correlational study of *A. baumannii* isolates between human, animal and environment such as pets, their owners and their environment should be performed to explore the transmission hypothesis of *A. baumannii*.

- Determination of genetic relatedness of *A. baumannii* should be conducted to elucidate the sources and the clonal spread of an endemic and an outbreak strains.
- Site-directed mutagenesis should be performed to identify the regulatory mutations responsible for transcription of RND efflux systems.
- Implementation of horizontal gene transfer mechanisms for antimicrobial resistance determinants in *A. baumannii* should be done to understand the dissemination of antimicrobial resistance among the organisms.

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APPENDIX

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APPENDIX A

Table A-1 Source of the *A. baumannii* clinical isolates from humans

Strains	Specimen
ABJ001	sputum
ABJ003	sputum
ABJ004	sputum
ABJ017	sputum
ABJ023	peritoneal fluid
ABJ025	sputum
ABJ029	sputum
ABJ032	sputum
ABJ033	blood
ABJ037	urine
ABJ039	sputum
ABJ042	sputum
ABJ045	wound (right ear)
ABJ047	blood
ABJ048	urine
ABJ049	urine
ABJ050	sputum
ABJ051	sputum
ABJ052	sputum
ABJ053	cerebrospinal fluid
ABJ054	blood
ABJ056	urine
ABJ057	blood
ABJ058	pleural fluid
ABJ059	wound
ABJ060	urine
ABJ061	wound
ABJ062	sputum
ABJ064	sputum
ABJ066	sputum
ABJ067	sputum
ABJ068	sputum
ABJ072	sputum
ABJ074	pus

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Strains	Specimen
ABJ076	sputum
ABJ077	blood
ABJ078	urine
ABJ079	sputum
ABJ080	penis
ABJ086	wound (right arm)
ABJ088	blood
ABJ090	sputum
ABJ091	sputum
ABJ092	sputum
ABJ094	wound (right leg)
ABJ098	sputum
ABJ099	sputum
ABJ101	wound (anus)
ABJ103	sputum
ABJ104	sputum
ABJ106	wound
ABJ111	sputum
ABJ112	sputum
ABJ114	wound (head)
ABJ117	wound (right hip)
ABJ118	wound
ABJ121	sputum
ABJ122	sputum
ABJ123	sputum
ABJ124	sputum
ABJ126	sputum
ABJ128	wound exudate
ABJ130	wound (right hip)
ABJ132	endotracheal tube
ABJ133	sputum
ABJ135	sputum
ABJ144	sputum
ABJ149	sputum
ABJ153	sputum
ABJ154	sputum
ABJ158	pus

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Strains	Specimen
ABJ159	wound (chest)
ABJ161	sputum
ABJ162	sputum
ABJ165	sputum
ABJ166	double lumen catheter
ABJ168	urine
ABJ171	wound (anus)
ABJ172	sputum
ABJ173	pus
ABJ174	sputum
ABJ177	sputum
ABJ178	sputum
ABJ179	wound
ABJ180	wound
ABJ181	sputum
ABJ182	sputum
ABJ184	sputum
ABJ186	sputum
ABJ187	wound (left leg)
ABJ188	sputum
ABJ193	sputum
ABJ195	sputum
ABJ199	penis
ABJ200	double lumen catheter
ABJ201	wound
ABJ202	sputum
ABJ203	sputum
ABJ205	blood
ABJ208	blood

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Table A-2 Source of the *A. baumannii* clinical isolates from animals

Strains	Species	Place of origin[a]
ABJ301	dog	Small Animal Teaching Hospital
ABJ302	dog	Small Animal Teaching Hospital
ABJ303	dog	Small Animal Teaching Hospital
ABJ304	cat	Small Animal Teaching Hospital
ABJ305	dog	Small Animal Teaching Hospital
ABJ306	dog	Small Animal Teaching Hospital
ABJ307	rabbit	Small Animal Teaching Hospital
ABJ308	rabbit	Small Animal Teaching Hospital
ABJ309	cat	Small Animal Teaching Hospital
ABJ310	cat	private animal hospital
ABJ311	dog	Small Animal Teaching Hospital
ABJ312	dog	Small Animal Teaching Hospital
ABJ313	rabbit	Small Animal Teaching Hospital
ABJ314	cat	Small Animal Teaching Hospital
ABJ315	dog	Small Animal Teaching Hospital
ABJ316	dog	Small Animal Teaching Hospital
ABJ317	dog	Small Animal Teaching Hospital
ABJ318	dog	Small Animal Teaching Hospital
ABJ319	cat	owner
ABJ320	dog	Small Animal Teaching Hospital
ABJ321	pig	pig farm
ABJ323	dog	Small Animal Teaching Hospital
ABJ324	cat	Small Animal Teaching Hospital
ABJ325	cat	Small Animal Teaching Hospital
ABJ326	dog	Small Animal Teaching Hospital
ABJ327	cat	Small Animal Teaching Hospital
ABJ328	dog	Small Animal Teaching Hospital
ABJ329	dog	Small Animal Teaching Hospital
ABJ330	rabbit	Small Animal Teaching Hospital
ABJ331	dog	owner

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APPENDIX B

Table B-1 Result of *in vitro* exposure experiment in the *A. baumannii* clinical isolates from humans and animals

	Strains	benzalkonium chloride		chlorhexidine		triclosan	
		Highest concentration (fold)	Presence of mutant derivative	Highest concentration (fold)	Presence of mutant derivative	Highest concentration (fold)	Presence of mutant derivative
Human clinical isolates (n=10)	ABJ058	0.25	-	0.38	-	1.90	-
	ABJ106	0.38	-	1.27	-	0.84	-
	ABJ114	0.25	-	0.38	-	164.21	+
	ABJ159	0.25	-	0.25	-	21.62	+
	ABJ174	0.25	-	0.25	-	32.44	+
	ABJ178	0.38	-	0.25	-	72.98	+
	ABJ184	0.38	-	0.25	-	1.90	-
	ABJ203	0.25	-	1.27	-	1.27	-
	ABJ205	0.56	-	0.25	-	1.90	-
	ABJ208	0.25	-	1.27	-	72.98	+
Animal clinical isolates (n=15)	ABJ302	0.84	-	1.27	-	21.62	+
	ABJ303	0.56	-	0.38	-	9.61	+
	ABJ308	0.56	-	0.56	-	0.38	-
	ABJ311	0.84	-	0.84	-	48.65	+
	ABJ316	0.25	-	1.27	-	21.62	+
	ABJ317	0.56	-	0.38	-	72.98	+
	ABJ319	0.25	-	1.27	-	9.61	+
	ABJ320	0.25	-	0.38	-	246.32	+
	ABJ321	0.56	-	0.84	-	164.21	+
	ABJ323	0.38	-	0.84	-	72.98	+
	ABJ324	0.56	-	0.38	-	72.98	+
	ABJ325	0.56	-	1.27	-	0.56	-
	ABJ328	0.25	-	0.25	-	0.38	-
	ABJ329	0.25	-	1.27	-	48.65	+
	ABJ331	0.56	-	0.56	-	246.32	+

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VITA

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