Detection of β-lactams resistance genes and integrons of *Acinetobacter baumannii* isolated from the clinical specimens of the patients admitted at Phramongkutklao hospital

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Abstract

Acinetobacter baumannii has emerged over a past decade as a leading cause of healthcare-associated infections (HAI) as well as community-acquired infections. *A. baumannii* is low grade pathogen but now become one of the most important pathogens in HAI, it's rapidly evolved from susceptible to multidrug resistance strain. The objectives of the study were to detect the β-lactams resistance genes and integrons of *A. baumannii* previously isolated from the clinical specimens of the patients admitted at Phramongkutklao hospital. 114 isolates of *A. baumannii* from clinical specimens of 80 patients admitted at Phramongkutklao hospital during January to March 2008 was obtained from the previous study. All of the *A. baumannii* clinical isolates showed 100% susceptible to colistin and tigecycline. In this study, 90.6% of *A. baumannii* isolates were multidrug resistant (MDR) organisms, which characterized by resistance to three or more different classes of antibiotics. In this study thirty isolates, were investigated for the presence of β-lactamase encoding genes on chromosome and plasmid of *A. baumannii* by PCR technique. The bla_{OXA-51} on chromosome and plasmid were the most commonly found *i.e.* 100% and 86.6% of thirty selected isolates, respectively. Detection of integron on chromosome and plasmid of *A. baumannii*. The integron class I were the most commonly found *i.e.* 30% and 43.3% of thirty selected isolates, respectively. The result of this study indicated that environmental isolates were less resistance to antibiotics. Therefore, patients, environment and ward cleaning could help to eliminate reservoir of *A. baumannii*, hence the spread of multiresistant genes. To control the emergence of the MDR- *A. baumannii* nosocomial infection, detection of β-lactams resistance genes and integrons could be used to screen the epidemic and endemic strains of MDR- *A. baumannii.*

Keywords: *Acinetobacter baumannii* / β-lactams resistance genes/ Integrons

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Introduction

Acinetobacter baumannii has emerged over a past decade as a leading cause of healthcare-associated infections (HAI) as well as community-acquired infections. *A. baumannii,* an opportunistic Gram-negative coccobacillus, is previously considered as a low grade pathogen but now become one of the most important pathogens in HAI, due to its worldwide spread in healthcare settings and it's rapidly evolved from susceptible to multidrug resistance strain (1). Increasing antimicrobial resistance among *A. baumannii* isolates are reported from all over the world. Recently carbapenems- and colistin- resistance *A. baumannii* have been emerged worldwide (2-6). A previous study in Thailand in the year 1998 showed that 4% of *Acinetobacter* spp. isolates were resistant to imipenem (7). Since then, the incidence of imipenem-resistant *Acinetobacter*

spp. has dramatically increased to over 50% (The National Antimicrobial Resistance Surveillance Center of Thailand, 2007). At Siriraj Hospital (2002), 57% of *A. baumannii* isolates were resistant to all available antimicrobial agents and overall mortality rate of the patients infected with pandrug-resistant *A. baumannii* was 54.7%. At Maharat Nakhon Si Thammarat hospital, MDR *A. baumannii* were a causative microorganism 77.3% of all *A. baumannii* ventilator associated pneumonia (VAP) reported in 2006 (8). β-lactams resistance mechanisms of *A. baumannii* are described as natural and acquired resistance. Two intrinsic β-lactamases namely AmpC cephalosporinase and OXA-51 serine type oxacillinase contribute to the natural resistance as their encoding genes are chromosomally embedded (9). Increase expression of *AmpC* gene and resistance to extended-spectrum cephalosporins are observed in the presence or an upstream IS element (IS*Aba1*) (3, 6). At Phramongkutklao Hospital, *A. baumannii* is the most common pathogens causing hospital associated infections such as VAP, catheter related blood stream infection (CR-BSI) and catheter associated urinary tract infection (CAUTI). Increasing number of MDR *A. baumannii* isolates in the hospital raised a concern in infection control and dissemination of MDR *A. baumannii* to the patients and hospital environment. The present study aims to detect the β-lactams resistance genes in A. *baumannii* previously isolated from the patients admitted at hospital by multiplex PCR. The results of this study will provide information on genetic markers of β-lactams resistance genes which indicate the virulence factors of *A. baumannii* isolates in the hospital and would help us to predict the impact of clinical outcomes and appropriately modify the treatment and infection control measures.

Methodology

114 isolates of *A. baumannii* from clinical specimens of 80 patients admitted at Phramongkutklao hospital during January to March 2008 was obtained from the previous study (10). All *A. baumannii* isolates were identified according to nosocomial surveillance system of Phramongkutklao hospital and determined the antibiotic susceptibility by disk diffusion technique according to CLSI guidelines 2012.

Antimicrobial susceptibility of *A. baumannii* will be performed by disc diffusion technique in accordance with guidelines established by Clinical and Laboratory Standards Institute (CLSI, 2012). The following antimicrobial discs will be included; Ticarcillin, Ceftriaxone, Ceftazidime, Amikacin, Imipenem, Meropenem, Levofloxacin, Colistin, Tigecycline and Cefoperazone/ sulbactam.

The genomic DNAs will be extracted using NucleoSpin® Tissue kit and following the manufacturer's instruction. Plasmid DNA purification will be extracted using NucleoSpin® Plasmid DNA purification kit by following the manufacturer's instruction. In this study thirty isolates were investigated for the presence of β-lactamase encoding genes by PCR technique. The primer sets for β-lactam resistance genes are shown in table 1.

Table 1. Primers used in PCR amplification

Primers	Target	Sequence(5´ to 3´)	Product size	Reference
AmpC F AmpC R	*ampC*	ACT TAC TTC AAC TCG CGA CG CCT TAA TGC GCT CTT CAT TTG G	773	11 12
PER F PER R	*bla*PER-1	CCT GAC GAT CTG GAA CCT TT TGG TCC TGT GGT GGT TTC	513	13
IMP F IMP R	*bla*IMP	GGAATAGAGTGGCTTAATTCTC CCAAACCACTACGTTATCT	188	14
VIM F VIM R	*bla*VIM	GAT GGT GTT TGG TCG CAT A CGA ATG CGC AGC ACC AG	390	14
OXA-23 F OXA-23 R	*bla*OXA-23	GAT CGG ATT GGA GAA CCA GA ATT TCT GAC CGC ATT TCC AT	501	14
OXA-40 F OXA-40 R	*bla*OXA-40 *bla*OXA-40	GGT TAG TTG GCC CCC TTA AA AGT TGA GCG AAA AGG GGA TT	246	14
OXA-51 F OXA-51 R	*bla*OXA-51	TAA TGC TTT GAT CGG CCT TG TGG TGG ATT GCA CTT CAT CTT GG	353	14

β-lactamase encoding genes detection by polymerase chain reaction

Amplification will be performed with 10 µl of this dilution as the DNA template. PCR conditions included 35 cycles of amplification under the following conditions: denaturation at 95°C for 30 s, annealing for 1 min at primer setspecific temperatures, and extension at 72°C for 1 min/kb product. Cycling was followed by a final extension at 72°C for 10 min. PCR products were resolved on 1.0% agarose gels, stained with ethidium bromide, and photographed with UV illumination (15).

DNA and plasmid template for multiplex PCR to detected integron in 30 isolates *A. baumannii* was prepared by NucleoSpin® Tissue kit. The primer sets for integrons are shown in table 2. (16).

Table 2. Primers integrons used in PCR amplification

Primers	Target	Sequence(5'to3')	Position	Product size
Int F IntR	*Int 1*	CAG TGG ACG TAA GCC TGT TC CCC GAG GCA TAG ACT GTA	2734-2751 2874-2891	160
Int2.R Int2.F	*Int2*	GTA GCA AAC GAG TGA CGA AAT G CAC GGA TAT GCG ACA AAA AGG T	11524-11545 12291-12312	788
Int3.R Int3.F	*Int3*	GCC TCC GGC AGC GAC TTT CAG ACG GAT CTG CCA AAC ATG ACT	738-758 1697-1717	979

Integron detection by multiplex PCR amplification

PCR amplification will be carried out in 50 µl volume. Each reaction will be contained 200 ng of purified DNA, 0.2 mM (each) deoxynucleotide triphosphate (dNTP), 1xThermoPol buffer, 1 U of *Taq* polymerase (NEB, MA, USA), and 200 nM of each primer. The sequence of the primers added in PCR. PCR amplification will be performed in a PTC-100 Peltier thermal cycler (MJ Research, MA, USA). Amplification products will be resolved by electrophoresis at 100 V for 50 min on 1% agarose gels in 1x Tris-Borate-EDTA (TBE) buffer, containing ethidium bromide (0.2 µg/ml) and visualize under UV light. All PCR amplification will be performed in duplicate. The amplification cycles will be seted as follows; initial denaturation at 95°C for 2 min follow by 35 cycles at 94°C for 1 min, 59°C for 1 min, 72°C for 1 min and a final extension at 72 °C for 5 min (16, 17).

Results

The Antimicrobial susceptibility of 10 antimicrobial agents against 106 *A. baumannii* clinical isolates were summarized in table 3. All of the A. *baumannii* clinical isolates showed 100% susceptible to colistin and tigecycline. *A. baumannii* clinical isolates were resistant to ceftriaxone 91.5%, ceftazidime 90.5%, ticarcillin 90.5%, meropenem 90.5%, imipenem 88.6%, levofloxacin 87.7%, cefoperazone/ sulbactam 67%, amikacin 38.6%. In this study, 90.6% (96/106) of *A. baumannii* isolates were multi drug resistant (MDR) organisms, which characterized by resistance to three or more different classes of antibiotics including 3rd generation of cephalosporin, beta-lactams, aminoglycosides and fluoroquinolone.

Table 3. Antimicrobial susceptibility test of A. *baumannii* isolated from the patients

Antimicrobial agent	Resistant No (%)	Intermediate No (%)	Susceptible No (%)
Ceftriaxone	97 (91.5)	9 (8.5)	-
Ceftazidime	96 (90.5)	-	10 (9.5)
Ticarcillin	96 (90.5)	-	10 (9.5)
Meropenem	96 (90.5)	-	10 (9.5)
Imipenem	94 (88.6)	1 (1)	11 (10.3)
Levofloxacin	93 (87.7)	2 (1.9)	11 (10.3)
Cefoperazone/sulbactam	71 (67)	21 (20)	14 (13)
Amikacin	41 (38.6)	7 (6.6)	58 (54.7)
Colistin	-	-	106 (100)
Tigecycline	-	-	106 (100)

Detection of β-lactamase encoding genes on chromosome and plasmid of *A. baumannii* were present in Table 4. The bla_{OXA-51} on chromosome and plasmid were the most commonly found *i.e.* 100% (30/30) and 86.6% (26/30) of thirty selected isolates, respectively. On chromosome can also be found bla_{AmpC} were identified in all isolates whereas none of them were positive for bla_{PER} and bla_{IMP}. In addition can also be found bla_{OXA-23} about 66.6% (20/30), bla_{VIM} about 33.3% (10/30) and bla_{OXA-40} about 10% (3/30) respectively. In the section on plasmid found first bla_{OXA-51} can also be found bla_{OXA-23} about 83.3% (25/30), $bla_{AmpC\ about}$ 53.3% (16/30), *blaVIM* about 33.3% (10/30), bla_{IMP} about 10% (3/30), bla_{OXA-40} about 3.3% (1/30) and results showed negative all of isolates for $bla_{PER.}$

Detection of integron on chromosome and plasmid of *A. baumannii* were present in Table 4. The integron class I on chromosome and plasmid were the most commonly found *i.e.* 30% (9/30) and 43.3% (13/30) of thirty selected isolates, respectively, whereas none of them positive for integron class II and class III on both chromosome and plasmid.

Table 4. β-lactam resistance genes and Integron

β-lactam resistance genes and Integron	Chromosome	Plasmid
bla_{PER}	0	0
bla_{IMP}	0	3 (10%)
bla_{VIM}	10 (33.3%)	10 (33.3%)
bla_{AmpC}	30 (100%)	16 (53%)
bla_{OXA-23}	20 (66.6%)	25 (83.3%)
bla_{OXA-40}	3 (10%)	1 (3.3%)
bla_{OXA-51}	30 (100%)	26 (86.6%)
Int I	9 (30%)	13 (43.3%)
Int II	0	0
Int III	0	0

Discussion and Conclusion

The impact of *A. baumannii* multidrug resistant on nosocomial infection causes an increasing problem in Phramongkutklao Hospital and tends to speed rapidly. Previous studies have shown that infected or colonized of *A. baumannii* patients, infection (90%, 72 of 80 cases), colonization (7.5%), and community-acquired infection (2.5%) with mortality rate of 50% (10). This study was aimed to detect the β-lactams resistance genes and integrons of *A. baumannii* previously isolated from the clinical specimens of the patients admitted at Phramongkutklao hospital.

The isolated *A. baumannii* in the present study were MDR- *A. baumannii* about 90.6% of all isolates. A report from Siriraj Hospital revealed the prevalence of MDR- *A. baumannii* to be 57.6% during 1996 and 1997 (18). In this study MDR- *A. baumannii* was defined as isolates that were resistant to 3rd generation of cephalosporin, β-lactams, aminoglycosides and fluoroquinolone. If we considered carbapenem-resistant isolates as MDR- *A. baumannii,* the proportion of MDR- *A. baumannii* would be similar, since carbapenem-resistant isolates were detected more than 89.6% of all isolates. In this study MDR- *A. baumannii* was defined as isolates that were resistant to all test antibiotics except colistin and tigecycline.

In the present study, OXA-51 and OXA-23 were the most predominant β-lactamases encoding genes. These enzyme groups have been known as a carbapenemases group and play an important role in human infection worldwide, with no exception for Thailand. Carbapenemases belongs to two molecular classes, class B (metallo β-lactamases) and class D (oxacillinases). Oxacillinases encoding genes are further divided into 4 groups, OXA-type β-lactamases was

discovered in an imipenem-resistant *A. baumannii* strain that was found to possess carbapenem hydrolysing activity. Imipenem resistance was subsequently demonstrated to be transferable and sequence analysis of the gene discovered that it encoded an unusual OXA-type enzyme (designated OXA-23) of Ambler molecular class D (19). OXA-51 was characterized from two imipenem-resistant *A. baumannii* clones isolated in Argentina (20). In this study detected $bla_{\text{OXA-51}}$ on chromosome and plasmid were the most commonly found *i.e.* 100% and 86.6% of thirty selected isolates, respectively and can also be found $bla_{\text{OXA-23}}$ on chromosome and plasmid about 66.6% and 83.3% of thirty selected isolates, respectively. Metallo β-lactamases genes like, *bla*IMP, *bla*VIM, *and bla*SIM are only present in *A. baumannii*. In this study detected bla_{VIM} about 33.3% on chromosome and bla_{VIM} about 33.3%, bla_{IMP} about 10% on plasmid.

AmpC β-lactamases (class C) was recently sequenced from a multi-resistant *A. baumannii* clinical strain (Ab RYC 52763/97) isolated during an outbreak in Spain. This constitutively expressed enzyme does not share strong similarity with Enterobacteriaceae AmpC cephalosporinases. As of yet there is no information about the regulation of *A. baumannii* AmpC expression (21). In this study detected can also be found bla_{AmpC} were identified in all isolates on chromosome and bla_{AmpC} about 53.3% on plasmid.

Class I integron is strongly associated with multi-resistance seen in MDR- *A. baumannii.* They are central in the worldwide problems of the antibiotic resistance. They are frequently associated with transmissible plasmids or transposons, the elements that facilitate their lateral transfer into a wide range of human pathogen. Class 1 integrons being most prevalent in clinical isolates, carrying single or multiple gene cassettes. Integron inserted genes encode for various antibiotic resistance mechanisms, including over 40 distinct genes, conferring resistance to aminoglycosides, β-lactams, chloramphenicol, macrolides, sulphonamides, antiseptics and disinfectants (22). This study investigated for the presence of integrons class I by PCR on chromosome and plasmid of *A. baumannii* were the most commonly found *i.e.* 30% and 43.3% of thirty selected isolates, respectively.

Antibiotic sensitivity pattern of microorganism may alert us to the emergence of a MDR- *A. baumannii* outbreak, but distinction between strains with slight differences in resistant profile as observed in this study (pattern 1-15) may be difficult. Antibiogram is phenotypic expression of the test isolates. The antibiogram might change overtime if they acquired resistant genes. In this

study the most prevalent pattern 1 which MDR- *A. baumannii.* It was possible that some of the resistant genes might be located on plasmids.

The contamination of MDR- *A. baumannii* in clinical specimens of patients with identical molecular type of the patients confirmed the importance of the environment as a reservoir in the spread of MDR- *A. baumannii* in the hospital. The result of this study indicated that environmental isolates were less resista nce to antibiotics. Therefore, patients, environment and ward cleaning could help to eliminate reservoir of *A. baumannii* which carrying integron or other mobile elements and transfer genes laterally, hence the spread of multiresistant genes. To control the emergence of the MDR- *A. baumannii* nosocomial infection, detection of β-lactams resistance genes and integrons could be used to screen the epidemic and endemic strains of MDR- *A. baumannii.*

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