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MULTIPLE COMBINATION PATTERNS OF OXA-TYPE CARBAPENEM-HYDROLYZING AND METALLO-B-LACTAMASES ENCODING GENES AMONG CLINICALLY ISOLATED *ACINETOBACTER BAUMANNII*.

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ABSTRACT

Acinetobacter baumannii has recently become one of the most important nosocomial pathogens worldwide due to its resistance to nearly all available antibiotic including carbapenems. The most common mechanisms of carbapenem resistance are acquired OXA-type carbapenem-hydrolyzing class D β-lactamases (CHDLs) and metallo-β-lactamases (MBLs). The study determined the presence of CHDLs and MBLs encoding genes among *A. baumannii* recovered from Ramathibodi Hospital, Mahidol University, Thailand during May 2008 to March 2010. A total of 353 isolates of non-susceptible carbapenems *A. baumannii* were examined by Double-disk synergy test (DDST) of phenotypic MBLs detection and the simplex PCR assay of CHDLs and MBLs encoding genes were confirmed the positive phenotypic isolates. Twenty-six isolates of *A. baumannii* were positive DDST phenotypic method and the $bla_{IMP-14a}$, bla_{OXA-23}-like, bla_{OXA-24}-like, and bla_{OXA-58}-like were detected. It is first reported in Southeast Asia that demonstrated the combination of various carbapenem resistant genes among *A. baumannii* isolates.

KEYWORDS: **Metallo-β-lactamases, Carbapenem-hydrolyzing class D β-lactamases, *Acinetobacter baumannii*, and Carbapenem.**

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INTRODUCTION

Acinetobacter baumannii is non-fermentative Gram-negative bacteria with coccobacilli form. It is an important hospital-acquired pathogen which has become more recognized due to the increase of resistance to nearly all classes and type of antibiotics including carbapenems [1,2,3]. The emergence of carbapenem-resistant *Acinetobacter* spp. has been reported worldwide [4,5,6]. Despite the existing of various carbapenem resistant mechanisms such as acquired OXA-type carbapenem-hydrolyzing class D β-lactamases (CHDLs), metallo-β-lactamases (MBLs), loss of outer membrane proteins (OMPs), and efflux pump overexpression have been reported in *A. baumannii* [7,8,9,10,11,12], only the first two mechanisms are commonly recovered [10]. The acquired OXA-type CHDLs producing isolates clustered in three major subfamilies; $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$ and $bla_{\text{OXA-58}}$ types while Class B MBLs; bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{GIM} and bla_{SPM} types are detected [8, 10,11,12,13,14]. Multidrug-resistant isolates of *A. baumannii* have been increasingly reported in various parts of the world including Asia [5,15]. The report from the SENTRY antimicrobial surveillance program in Asia-Pacific Nations (APAC) region demonstrated high recovery rates of carbapenem-resistance in *A. baumannii* [16]. Reports from 6 Asian nations; China, Korea, Singapore, India, Hong Kong, and Thailand revealed that the $bla_{\text{OXA-23}}$ gene are highly disseminated among *A. baumannii* isolates [16,17,18,19,20]. The MBLs encoding of carbapenem-resistance *A. baumannii* isolates, on the other hand, are not as highly recovered as the $bla_{\text{OXA-23}}$ gene. It has been scattered recovered in different countries. For instance, the $bla_{\text{IMP-4}}$ was detected in *A. baumannii* isolated from Philippines while $bla_{\text{VIM-2}}$ was detected from Korea [16,20]. In addition, *A. baumannii* that carrying $bla_{\text{SIM-1}}$ gene was firstly identified from and spread only in Seoul [20]. Nevertheless, according to high diversity of CHDLs and MBLs encoding genes in *A. baumannii*, varieties of carbapenem MIC level were observed [8, 14]. Because of the mobile genetic structures of CHDLs and MBLs, that are succeed in spreading, it is challenging to detect the CHDLs and MBLs positive producing strains in routine microbiology laboratories. In this study, we evaluated the occurrence and dissemination of CHDLs and MBLs encoding genes among *A. baumannii* recovered from a 1,000-beds hospital, in Bangkok, Thailand.

MATERIALS AND METHODS

Bacterial isolates

A total of 353 clinical *A. baumannii* isolates were collected from routine samples sent to Microbiology Laboratory, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, during May 2008 to March 2010. Isolates which showed non-susceptible to either imipenem or meropenem or both antibiotics were selected. All isolates were identified using the Microscan WalkAway automated system (Siemens, USA) and the 16s rRNA sequencing method was used for confirmation of selected isolates.

Antimicrobial susceptibility testing

Antimicrobial susceptibility test of all isolates were initially performed by the routine disk diffusion method following the CLSI guideline [21]. The minimum inhibitory concentrations (MICs) of imipenem, meropenem, and doripenem and others antibiotics were examined by using Microscan® Dried Gram Negative MIC/Combo Panels (Siemens, USA). For more details of the MICs of the above carbapenems, the E-test was performed.

Phenotypic detection of MBLs

Double-disk synergy test (DDST) for detecting MBLs producer was developed and modified following Arakawa's publication [22]. Three chelating agents, 1 µl of undiluted 2-mercaptopropionic acid (2-MPA), 5 µl of 0.5 M ethylenediaminetetraacetic acid (EDTA), and 10 µl of 0.5 M dipicolinic acid (DPA) were used as MBL producing inhibitors on the phenotypic detection [23,24]. The presence of the synergistic inhibition pattern indicated the strain as positive for MBLs.

MBLs- and CHDLs-encoding genes detection

The simplex PCR assay was used to detect carbapenem resistant genes from the positive

phenotypic isolates. The MBLs and CHDLs primers as well as the insertion IS*Aba*1 gene primer were established for the existence of resistant genes. The details of primers are shown in Table 1. The PCR products were analyzed for their sizes and amount. In addition, gene cassette analysis was performed with primer 5'-CS and 3'-CS and the structure of the variable region of integron cassettes containing MBL gene were determined by PCR mapping [25,26]. The PCR amplicons from the gene cassettes analysis were purified by using the HiYield™ Gel/PCR DNA Extraction Kit (RBC bioscience, Taiwan). The products were sent to the 1st Base Laboratories, Malaysia bi-direction DNA sequencing. The nucleotide sequences were analyzed and compared by using the BLAST program software available at http://www.ncbi.nlm.nih.gov/blast/.

Random Amplified Polymorphic DNA (RAPD) method

The positive isolates for MBLs and CHDLs genes by PCR were analyzed by random amplified polymorphic DNA technique to generate a DNA fingerprint. The RAPD analysis was performed with the primer 5´-GGCATCCAAGCAGCAAG-3´ [27].

Cloning method and antibiotic susceptibility test of transformant

The PCR products of MBL gene cassette from gene cassettes analysis method were purified and ligated into the pDrive cloning vector (Qiagen, Germany). These vectors were transformed into the competent *Escherichia coli* cells (Qiagen EZ competent cell). The transformants were selected by LB agar plates containing 100 µg/ml ampicillin. The MIC of carbapenems of transformants and competent cells were examined and compared.

Table 1
The primers for amplification of the CHDLs and MBLs genes

Primer Name	Sequence 5'-3'	Product size (bp)
IMP-F-5-7-9-14-15-25	AAT TGA GAA GCT TGA CGA AGG	406
IMP-R-5-7-9-14-15-25	TTC AGG TAG CCA AAC CAC TAC G	
VIM-F	GTT TGG TCG CAT ATC GCA AC	512
VIM-R	GGG TAG TGT TGT TGA ATC CGC TCA A	
OXA-23PF	GAT GTG TCA TAG TAT TCG TCG TTC	1024
OXA-23PR	ACA ACA ACT AAA AGC ACT GT	
OXA-51F	AAC AAG CGC TAT TTT TAT TTC AG	641
OXA-51R	CCC ATC CCC AAC CAC TTT T	
OXA-58F	AGT ATT GGG GCT TGT GCT	453
OXA-58R	AAC TTC CGT GCC TAT TTG	
OXA-24F	ATG AAA AAA TTT ATA CTT CCT ATA TTC AGC	825
OXA-24R	TTA AAT GAT TCC AAG ATT TTC TAG C	
ISAba1F	CAT TGG CAT TAA ACT GAG GAG AAA	451
ISAba1R	TTG GAA ATG GGG AAA ACG AA	
5´CS	GGC ATC CAA GCA GCA AG	variable
3´CS	AAG CAG ACT TGA CCT GA	

RESULTS

Antimicrobial susceptibility testing

All 353 tested isolates of *A. baumannii* revealed resistant to meropenem. Nearly all of these isolates also showed resistant to imipenem except isolate no.1 and 2, which were susceptible and intermediate susceptible to this drug, respectively.

Phenotypic detection of MBLs.

The Double-disk synergy test of the isolates against three individual chelating agents; 1 µl of undiluted 2-MPA, 5 µl of 0.5 M EDTA, and 10 µl of 0.5 M DPA revealed that only 26 isolates (7.37%) were phenotypic positive. In addition, all isolates were found to be positive by all three agents (Fig 1).

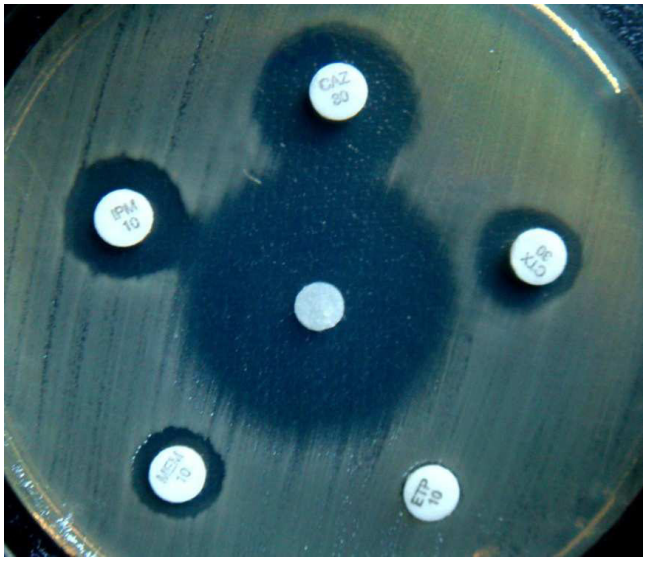

Figure 1
Phenotypic detection of MBLs showing the positive DDST of A. baumannii carrying bla$_{IMP-14a}$ and bla$_{OXA-23}$-like.

Detection of MBLs- and CHDLs-encoding genes.

The 26 phenotypic positive isolates were determined for the CHDLs (*bla*$_{OXA-23}$-like, *bla*$_{OXA-24}$-like, *bla*$_{OXA-51}$-like and *bla*$_{OXA-58}$-like) and MBLs (*bla*$_{IMP}$-like and *bla*$_{VIM}$-like) genes by simplex PCR using the specific group primer (Table 1). The results demonstrated that all of 26 isolates were found to harbor more than one resistant gene with at least one of CHDLs genes containing. The *bla*$_{OXA-23}$-like was the most predominated gene found in 22 (84.62%) isolates while *bla*$_{OXA-51}$-like, *bla*$_{OXA-24}$-like and *bla*$_{OXA-58}$-like were found in 18 (69.23%), 13 (50%) and 2 (7.69%), respectively. Only 8 isolates (30.77%) were found to carry *bla*$_{IMP}$-like gene. The DNA sequencing results of these 8 purified amplicons revealed as the *bla*$_{IMP-14a}$ gene. All of the above *bla*$_{IMP-14a}$ encoding isolates were also found to contain other CHDLs-encoding genes. Among these, 7 of 8 (87.5%) isolates harbored both of *bla*$_{IMP-14a}$ and *bla*$_{OXA-23}$-like while the other isolate carried *bla*$_{IMP-14a}$ and *bla*$_{OXA-58}$-like genes (Table 2). In contrast, the *bla*$_{VIM}$-like gene was not detected in any isolates.

Table 2
MBLs and CHDLs encoding genes detected among 26 positive phenotypic method A. baumannii isolates.

Isolates (no. of isolates)	CHDLs -encoding genes detection						
	bla$_{OXA-23}$-like only	*bla*$_{OXA-58}$-like only	*bla*$_{OXA-23}$-like & *bla*$_{OXA-51}$-like	*bla*$_{OXA-24}$-like & *bla*$_{OXA-51}$-like	*bla*$_{OXA-23}$-like & *bla*$_{OXA-24}$-like & *bla*$_{OXA-51}$-like	*bla*$_{OXA-24}$-like & *bla*$_{OXA-51}$-like & *bla*$_{OXA-58}$-like	IS*Aba*1
bla$_{IMP-14a}$-positive (8)	7	1	0	0	0	0	8
bla$_{IMP-14a}$ negative (18)	0	0	5	2	10	1	18
Total (26)	7	1	5	2	10	1	26

All of the 18 *bla*$_{IMP-14a}$ negative isolates were found to carry more than one CHDLs-encoding gene. Interestingly, each individual isolate harbored the *bla*$_{OXA-51}$-like gene, in contrast, this resistant gene could not be detected in any of *bla*$_{IMP-14a}$ positive isolates. In addition to the *bla*$_{OXA-51}$-like gene, 11 of 18 (61.11%) isolates carried at least other two resistant genes. Among these, 10 isolates contained *bla*$_{OXA-23}$-like and *bla*$_{OXA-24}$-like , while the other isolate carried *bla*$_{OXA-24}$-like and *bla*$_{OXA-58}$-like genes. The other 7 isolates were found to carry two other CHDLs-encoding genes. The details showed in Table 2. In addition, IS*Aba*1 gene was positive in all 26 isolates. However,

the association of IS*Aba1* and CHDLs-encoding gene for expression was not performed (Table 2). The RA-41520608 *A. baumannii* strain containing *bla*$_{IMP-14a}$ and *aac(6')* genes was selected for the gene cassette analysis. The strain was demonstrated to harbor class I integron and contained a 1.3 kb gene cassette array with *attI1, bla*$_{IMP-14a}$ and *aac(6')* (GenBank accession number GQ302618) (Fig 2).

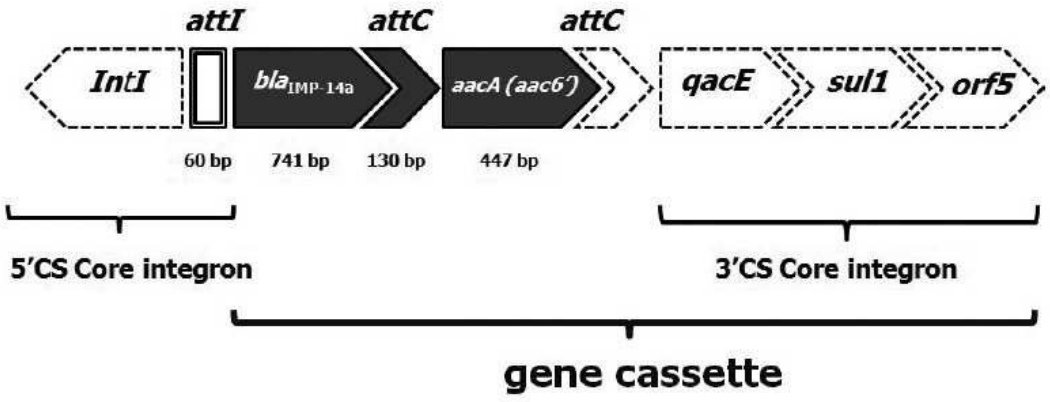

Figure 2
Structure of gene cassette containing attI1, bla$_{IMP-14a}$ and aac(6') gene from RA-41520608 A. baumannii.

MICs of carbapenem in A. baumannii strains carrying MBLs- and CHDLs - encoding genes.

The MICs of the above resistant isolates were determined by E-test. The results revealed variety of MIC ranges among different groups of organism (Table 3). With all combined results of resistant genes and MIC ranges, the data suggested that the highly carbapenem resistant outcome was mainly influenced by the *bla*$_{OXA-23}$-like gene. All of the isolated that contained *bla*$_{OXA-23}$-like gene revealed high MIC levels in all tested carbapenems (Table 3). In contrast, the *bla*$_{OXA-24}$-like and *bla*$_{OXA-51}$-like gene did not show much effect to the MIC level. The MICs of all carbapenems remained low. The combination of *bla*$_{OXA-14a}$ and *bla*$_{OXA-58}$-like gene showed minimal effect on imipenem and doripenem, while a slight increase of MIC level (4 ug/ml) occurred in meropenem. It remains unclear whether which of the above genes was responsible for the outcome. Although the *bla*$_{OXA-58}$-like in combination with *bla*$_{OXA-14a}$ gene as well as the *bla*$_{OXA-24}$-like plus *bla*$_{OXA-51}$-like genes did not reveal highly resistant, but interestingly, by adding the *bla*$_{OXA-58}$-like gene to other two CHDL genes (*bla*$_{OXA-24}$-like and *bla*$_{OXA-51}$-like) high level of carbapenem resistance occurred (Table 3).

Table 3
The MICs of carbapenems of A. baumannii isolates harboring MBLs and CHDLs genes.

	MICs Range (µg/ml)*					
Carbapenems	*bla*$_{IMP-14a}$ & *bla*$_{OXA-23}$-like	*bla*$_{IMP-14a}$ & *bla*$_{OXA-58}$-like	*bla*$_{OXA-23}$-like & *bla*$_{OXA-51}$-like	*bla*$_{OXA-24}$-like & *bla*$_{OXA-51}$-like	*bla*$_{OXA-23}$-like & *bla*$_{OXA-24}$-like & *bla*$_{OXA-51}$-like	*bla*$_{OXA-24}$-like & *bla*$_{OXA-51}$-like & *bla*$_{OXA-58}$-like
Imipenem	6 - >32	0.5	8 - >32	0.19 - 1	8 - >32	>32
Meropenem	>32	4	12 - >32	0.5 - 1.5	12 - >32	>32
Doripenem	8 - >32	0.75	8 – 16	0.125 - 1.5	8 - >32	>32

MICs range of A. baumannii isolates harboring MBLs and CHDLs genes were examined by E-test.

Random Amplified Polymorphic DNA (RAPD) in MBL positive isolates.

Eight isolates that carried *bla*$_{IMP-14a}$ gene were analyzed by random amplified polymorphic DNA technique for their DNA fingerprint. Severn isolated contained the similar resistant genes while the other isolate was carrying the *bla*$_{OXA-58}$-like instead of *bla*$_{OXA-23}$-like gene. The result of RAPD analysis revealed similar pattern in all isolates (Fig 3).

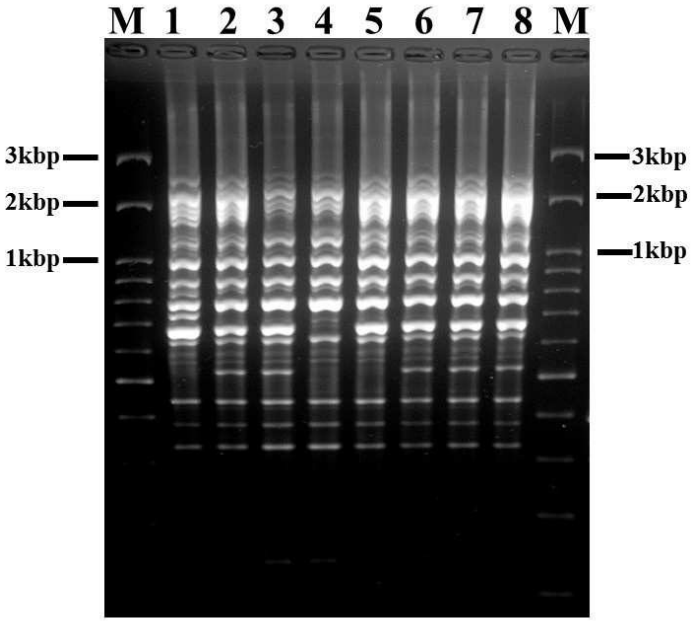

Figure 3

The pattern of RAPD of A. baumannii carrying bla$_{IMP-14a}$ + bla$_{OXA-23}$-like and bla$_{IMP-14a}$ + bla$_{OXA-58}$-like. (Lane 1; bla$_{IMP-14a}$ + bla$_{OXA-58}$-like , Lane 2-8; bla$_{IMP-14a}$ + bla$_{OXA-23}$-like)

Cloning method and antibiotic susceptibility test of transformant.

The gene cassette of RA-41520608 *A. baumannii* strain that carrying *attI1, bla*$_{IMP-14a}$ and *aac(6')* gene was purified and ligated into the pDrive cloning vector. These recombinant plasmids were then transformed into *E. coli* M15. Colonies of *E. coli* M15 containing recombinant plasmid (p4152-IMP-14a) were selected by LB agar plates containing 100 µg/ml ampicillin. The MICs of carbapenems of *E. coli* M15 harboring p4152-IMP-14a and *E. coli* M15 competent cells were examined and compared. *E. coli* M15 harboring p4152-IMP-14a that carrying *attI1, bla*$_{IMP-14a}$ and *aac(6')* gene showed increased MICs of carbapenem, ceftazidime, cefotaxime, and cefepime. The index strain, RA-41520608 *A. baumannii* remained highly resistant to carbapenem (Table 4).

Table 4

The MICs of antimicrobial agents of RA-41520608 A. baumannii strain and E. coli M15 harboring p4152-IMP-14a.

	MICs (µg/ml)*		
Antibiotic agents	RA-41520608	*E. coli* M15 harboring	*E. coli* M15
	A. baumannii	p4152-IMP-14a	
Imipenem	>32	>8	≤1
Meropenem	>32	8	≤1
Ertapenem	>32	4	≤0.5
Ceftazidime	>16	>16	≤2
Cefotaxime	>32	>32	≤2
Cefepime	>32	>32	≤2

Tests were examined by using Microscan® Dried Gram Negative MIC/Combo Panels.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this study are listed in the GenBank accession number GQ302618.

DISCUSSION

Carbapenem resistance in *A. baumannii* has been increasingly reported worldwide, especially in Asia[10,15]. The report from the SENTRY antimicrobial surveillance program in Asia-Pacific Nations (APAC) region, showed that 42.3% of clinical isolated were non-susceptible to imipenem or meropenem[16]. The carbapenem resistance mainly associated with carbapenemase production. The production of acquired OXA-type carbapenem-hydrolyzing class D β-lactamases (CHDLs), and metallo-β-lactamases (MBLs) are the most common mechanisms[10]. The prevalence of MBLs production by *A. baumannii* was very low, however, varieties of resistant genes including IMP-1, -2, -4, -5, -6, -11, VIM-2 and SIM- were found[6,10,15,16,20]. In addition to the MBLs associated resistant mechanisms, the OXA type including OXA-23, OXA-24, OXA-58-like had spread in CHDLs enzymes producing *A. baumannii*[16,17,18,19,20]. In this study, only 2.27% (8/353 isolates) of *A. baumannii* carried both of MBLs and CHDLs genes ($bla_{\text{IMP-14a}}$ and $bla_{\text{OXA-23}}$-like or $bla_{\text{OXA-58}}$-like). Furthermore, 5.1% (18/353 isolates) harbored CHDLs genes ($bla_{\text{OXA-23}}$-like, and $bla_{\text{OXA-24}}$-like or $bla_{\text{oxa-58}}$-like). Although these isolates contained only the CHLDs genes, they were detected positive in Double-disk synergy test for MBLs. This indicated that false positive results of phenotypic test among CHLDs producing strains could be observed. One of the explanation to this false positive results was the defection of cell permeability and the disruption of OXA dimmers[28, 29].

In Thailand, only a few reports have been established on the CHLDs producing *A. baumannii*. Mendes et al. reported the existence of $bla_{\text{OXA-23}}$-like, $bla_{\text{OXA-24}}$-like, $bla_{\text{OXA-58}}$-like, $bla_{\text{OXA-23}}$-like + $bla_{\text{OXA-58}}$-like, and $bla_{\text{OXA-24}}$-like + $bla_{\text{OXA-58}}$-like in *A. baumannii*. Additionally, Niumsup *et al* demonstrated the presence of $bla_{\text{OXA-23}}$-like in carbapenem-resistance *A. baumannii*[16, 30]. In 2010, Thapa *et al* found that $bla_{\text{OXA-23}}$-like were determined in clinical isolates of carbapenem-resistant *A. baumannii*[31]. Apart from the above resistant encoding genes, the $bla_{\text{OXA-51-like}}$ gene was found in nearly 70% of tested isolates. High prevalence of this gene was reported from various parts of the world[32, 33, 34], however it had yet reported in previous studies from Thailand. Conversely, MBLs producing in carbapenem-resistance *A. baumannii* has yet been reported in the region. To our knowledge, this study not only being the first report of the existence of $bla_{\text{IMP-14a}}$ in *A. baumannii,* but also the coexistence of $bla_{\text{IMP-14a}}$ with CHLDs genes; the $bla_{\text{OXA-23-like}}$ and $bla_{\text{IMP-14a}}$ with $bla_{\text{OXA-58-like}}$. Although the $bla_{\text{IMP-14}}$ gene had been deposited in GenBank with accession numbers AY553332, FN 397627, and FJ267651 from Norway and Thailand [35], these genes were detected in *Pseudomonas aeruginosa*. In this study, RA-41520608 *A. baumannii* strain (GenBank No. GQ302618) was represented by the $bla_{\text{IMP-14a}}$. Gene cassette of this strain contained class I integron with *attI1,* $bla_{\text{IMP-14a}}$ and *aac(6')* which indicated a high resistant to carbapenem. The MICs of RA-41520608 *A. baumannii* strain for imipenem, meropenem, and doripenem were >32 µg/ml. In RAPD analysis, one identical pattern from 8 isolates of *A. baumannii* strain that carried $bla_{\text{IMP-14a}}$ was shown. These isolates were collected from 3 wards. Six isolates were from ICU ward on the 9th floor, 1 isolate was from ICU on the 3rd floor in the same building while the other isolate was from a ward in another building. The results demonstrated that some clones of carbapenem resistance in *A. baumannii* strains had been spreading in the hospital. Previously, the clonal outbreaks of *A. baumannii* were reported in many countries in Asia including China, Korea, and Thailand[19,31,36] .

CONCLUSION

The carbapenem resistant *A. baumannii* isolates are emerging worldwide. The CHDLs

and MBLs are the most common mechanisms in carbapenem resistant *A. baumannii*. Nevertheless, to our knowledge, this is the first report from Southeast Asia of mixed combinations of MBLs and CHDLs, and the multiple CHDLs genes isolated among clinically isolated *A. baumannii*. Further establishment of these genes in larger scale will provide a clearer picture of the distribution of carbapenem resistant *A. baumannii* in the region.

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