EMERGENCE OF CO-CARBAPENEMASE GENES, BLA_{OXA23}, BLA_{VIM} AND BLA_{NDM} IN CARBAPENEM-RESISTANT *ACINETOBACTER BAUMANNII* CLINICAL ISOLATES

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Abstract. This study investigated presence of carbapenemase genes among carbapenem-resistant *Acinetobacter baumannii* (CRAB) clinical isolates and their clonal relationships. Fifty-six CRAB isolates were collected from patients admitted to Hua Hin Hospital, Prachuap Khiri Khan, Thailand. PCR amplification and DNA sequencing were used to identify bla_{OXA23}, bla_{OXA40}, bla_{OXA58}, bla_{VIM}, bla_{SIM} and bla_{NDM}. Clonal relationship was explored using repetitive element palindromic (REP)-PCR. Plasmid profiling was obtained from *Eco*RI-digested fragments. The CRAB isolates were classified by REP-PCR into 12 groups, with 71% belonging to group I, which was associated with the presence of bla_{OXA23}. Co-existence of bla_{OXA23} + bla_{VIM2} (n = 20), bla_{OXA23} + bla_{NDM1} (n = 2), bla_{VIM2} + bla_{NDM1} (n = 1), and bla_{OXA23} + bla_{VIM2} + bla_{NDM1} (n = 1) were discovered. The emergence of CRAB carrying multiple types of carbapenemase genes (the first such report in Thailand) is a worrying phenomenon and public health measures should be put in place to prevent any serious nosocomial infection and to contain the spread of such CRAB genotypes.

Keywords: *Acinetobacter baumannii*, bla_{OXA23}, bla_{VIM2}, bla_{NDM1}, carbapenemase gene

INTRODUCTION

To date, nosocomial infection with multidrug-resistant (MDR) pathogens, especially *Acinetobacter baumannii*, has rapidly expanded in various parts of the world (Durante-Mangoni and Zarrilli, 2011). The National Antimicrobial Resistant Surveillance of Thailand (NARST) revealed that carbapenem-resistant *A. baumannii* (CRAB) has increased from 5.8% in 2000 to 66.6% in 2015 (NARST, 2016).

Typically, CRAB is resistant to carbapenem through the presence of drug-inactivating enzymes, carbapenemases, which are classified into two groups, namely, class B β-lactamase (metallo-β-lactamase) and class D β-lactamase (OXA family) (Poirel and Nordmann,

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2006). Metallo-β-lactamase are carried via mobile genetic elements (Poirel and Nordmann, 2006; Tsakris *et al*, 2006; Chen *et al*, 2011a). For example, bla_{VIM2} cassettes are inserted into class 1 integron in CRAB isolated from patients in Greece (Tsakris *et al*, 2006). More recently, bla_{NDM} was discovered in CRAB (Chen *et al*, 2011b; Johnson and Woodferd, 2013). This gene, together with other resistance genes, usually is located on plasmids, which are easily transferred among intra- and inter-species of bacteria (Walsh *et al*, 2011).

Previous studies in Thailand reported CRAB isolates carrying bla_{OXA23} (Mendes *et al*, 2009; Niumsup *et al*, 2009; Higgins *et al*, 2010) and one study revealing an isolate carrying bla_{OXA40} (Santimaleeworagun *et al*, 2014). This study investigated the presence of carbapenemase genes in nosocomial CRAB isolates to understand evolution of antibiotic resistance genes.

MATERIALS AND METHODS

Bacterial isolates

Clinical *A. baumannii* isolates were collected from patients admitted to Hua-Hin Hospital, Prachuap Khiri Khan Province, Thailand during 2013 - 2015. A CRAB isolate is defined as showing either imipenem or meropenem resistance by disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) standard (CLSI, 2015). All CRAB isolates were kept in tryptic soy broth (TSB) (OXOID, Hampshire, England) containing 15% glycerol at -20°C until used.

The institutional review board of the Faculty of Pharmacy, Silpakorn University approved the research protocol with a waiver for informed consent (no. 20-2558).

Carbapenemase gene identification

Genomic DNA of *A. baumannii* isolates were extracted using GeneJET genomic DNA purification kit (Thermo Scientific, Waltham, MA). Carbapenemase genes, bla_{OXA23}, bla_{OXA40}, bla_{OXA58}, bla_{IMP}, bla_{VIM}, bla_{SIM} and bla_{NDM} were detected by PCR, using specific primers and annealing temperatures indicated in Table 1. The 15 μl PCR mixture was composed of 2 μl of DNA, 0.4 μl of 20 μM each forward and reverse primers, 7.5 μl of PCR master mix kit (JumpStart Red Taq® Ready Mix; Sigma, St Louis, MO) and 4.7 μl of DNAase-free water. Thermocycling was conducted in Biometra TGradient Thermocycler (Biometra, Göttingen, Germany) as follows: 95ºC for 2 minutes; 30 cycles of 95ºC for 30 seconds, specific annealing temperature for 30 seconds, 68ºC for 1.5 minutes; final step at 68ºC for 5 minutes (Santimaleeworagun *et al*, 2014). Amplicons were gel-purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid, New Taipei City, Taiwan) and directly sequenced (1st Base, Selangor, Malaysia).

Repetitive element palindromic (REP) typing

Repetitive element palindromic PCR was used to determine clonal relationship of CRAB isolates. PCR targets were repetitive extra-genic palindromic sequences dispersed in non-coding sequences. REP primers are shown in Table 1. The 15 μl PCR mixture was composed of 1 μl of DNA, 0.4 μl of 20 μM each forward and reverse primers, 7.5 μl of PCR master mix kit (JumpStart Red Taq® Ready Mix) and 5.7 μl of DNAase-free water. Thermocycling was conducted in Biometra TGradient Thermocycler as follows: 94ºC for 10 minutes; 30 cycles of 94ºC for 1 minute, 45 ºC for 1 minute, and 72ºC for 2 minutes; and a final step at 72ºC for 16 minutes (Santimaleeworagun *et al*, 2014). Amplicons were separated by 2% aga-

Table 1
Primers, amplicon sizes and annealing temperatures used for PCR of carbapenemase genes.

Gene	Primer sequence	Amplicon size (bp)	Annealing temperature (°C)
	F = forward; R = reverse		
bla_{IMP}	F-5′CTRCCGCAGSAGMGBCTTTG3′ R-5′AACCAGTTTTGCHTTACCAT3′	232	57
bla_{OXA23}	F-5′GGAATTCCATGAATAAATATTTTA3′ R-5′GGATCCCGTTAAATAATATTCAGC3′	822	42
bla_{OXA40}	F-5′GGAATTCCATGAAAAAATTTATAC3′ R-5′GGATCCCGTTAAATGATTCCAAGA3′	828	45
bla_{OXA58}	F-5′GGAATTCCATGAAATTATTAAAAA3′ R-5′GGATCCCGTTATAAATAATGAAAA3′	843	45
bla_{VIM}	F-5′GGAATTCCATGTTAAAAGTTATTA3′ R-5′GGATCCCGCTACTCGGCGACTGAG3′	390	57
bla_{SIM}	F-5′GGAATTCCATGAGAACTTTATTGA3′ R-5′GGATCCCGTTAATTAATGAGCGGC3′	741	47
bla_{NDM}	F-5′GGTTTGGCGATCTGGTTTTC3′ R-5′CGGAATGGCTCATCACGATC3′	621	57
bla_{KPC}	F-5′CGTCTAGTTCTGCTGTCTTG3′ R-5′CTTGTCATCCTTGTTAGGCG3′	798	57

B = Cytosine or Guanine or Thymine, H = Adenine or Cytosine or Thymine M = Adenine or Cytosine, R = Adenine or Guanine, S = Guanine or Cytosine.

rose gel-electrophoresis and visualized by ethidium bromide staining. If there are more than 3 bands different among clones, they are classified as belonging to different clones.

Plasmid profiling

DNA plasmids were extracted from *A. baumanii* isolates using GeneJet Plasmid Miniprep Kit (Thermo Scientific), digested with *Eco*RI (New England BioLabs, Ipswich, MA) and analyzed by 1% agarose gel-electrophoresis.

RESULTS

Identification of carbapenemase genes

Carbapenemase genes, bla_{OXA23}, bla_{OXA40}, bla_{OXA58}, bla_{VIM}, bla_{SIM} and $bla_{NDM,}$ were detected by PCR, and the identity of the amplicons were confirmed by direct DNA sequencing. Among 56 isolates, 89% were CRAB positive and carried carbapenemase genes (Table 2). There were CRAB isolates carrying more than a single carbapenemase gene: 19 isolates with co-existing bla_{OXA23} and bla_{VIM2}, 2 with co-existing bla_{OXA23} and bla_{NDM1}, and 1 with co-existing bla_{VIM2} and bla_{NDM1}. It is of interest to note that one isolate, CRAB45, contained bla_{OXA23}, bla_{VIM2} and bla_{NDM1}.

Clonal relationship classified by REP-PCR

There were 12 detectable patterns for REP-PCR (Fig 1), 73% of which were of Pattern I (Table 2). Interestingly, the majority of isolates harboring bla_{OXA23}, $bla_{OXA23} + bla_{VIM2}$, $bla_{OXA23} + bla_{NDM1,}$ bla_{VIM2}

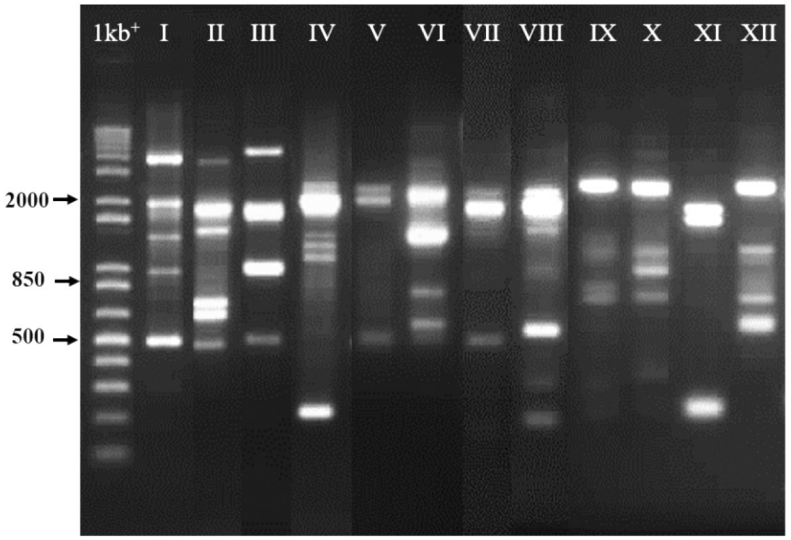

Fig 1–REP-PCR patterns of CRAB isolates. PCR protocol is described in Materials and Methods. Lane 1kb+, DNA size markers (1 Kb Plus DNA Ladder, Invitrogen, USA); lane I – XII, REP patterns.

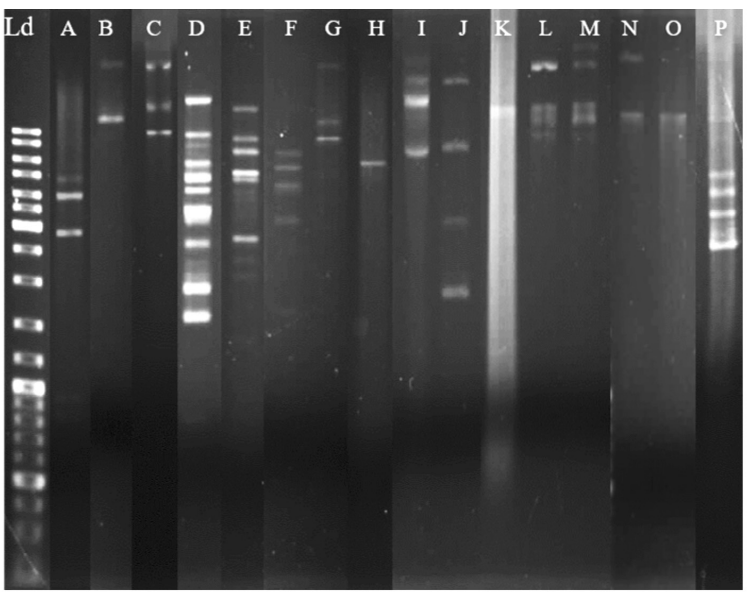

Fig 2–Plasmid profile of CRAB isolates. Plasmid profiling protocol is described in Materials and Methods. Lane Ld, DNA size markers (GeneRuler DNA Ladder Mix, Thermo Scientific, USA); lanes A - P, plasmid patterns.

+ bla_{NDM1}, and bla_{OXA23} + bla_{VIM2} + bla_{NDM1} belonged to REP Pattern I, whereas isolates carrying bla_{VIM2} belonged to various REP patterns.

Plasmid profile

Plasmid profiling was used for identifying genetic relationship among CRAB isolates. Sixteen patterns of plasmids were identified (Fig 2). Plasmid pattern A was common in only single bla_{OXA23}-positive CRAB isolates, whereas plasmid pattern B and C were widespread among CRAB isolates carrying bla_{OXA23} + bla_{VIM2} (Table 2) . In addition, plasmid pattern C was present in isolates carrying bla_{OXA23} + bla_{NDM1}, and bla_{OXA23} + bla_{VIM2} + bla_{NDM1}. It was noteworthy that both plasmid patterns B and C had one common plasmid band, and that plasmid patterns A, B, C were absent in single bla_{VIM}-positive CRAB isolates.

DISCUSSION

In this study, we found several carbapenemase genes including bla_{OXA23}, bla_{VIM2} and bla_{NDM1} among clinical *A. baumannii* isolates. The majority of CRAB isolates contained bla_{OXA23}, in agreement with previous studies in various

Table 2
Genetic relatedness of CRAB isolates carrying carbapenemase gene(s).

Carbapenemase genotype	No. of isolates	REP-PCR pattern (*n*)	Plasmid profile pattern (*n*)
bla_{OXA23}	20	I (17)	A (15), D (1), G (1)
		III (1)	F (1)
		VII (1)	n/a (1)
		VIII (1)	D (1)
bla_{VIM}	7	I (2)	D (1), L (1)
		III (1)	n/a (1)
		IV (2)	n/a (1), K (1)
		V (1)	n/a (1)
		VI (1)	I (1)
bla_{OXA23}+bla_{VIM}	19	I (18)	B (6), C (10), M(1), R (1)
		II (1)	B (1)
bla_{OXA23}+bla_{NDM}	2	I (2)	C (2)
bla_{VIM}+bla_{NDM}	1	I (1)	J (1)
bla_{OXA23}+bla_{VIM}+bla_{NDM}	1	I (1)	C (1)
None	6	IV (2)	H (1), VI (n/a)
		IX (1)	N (1)
		X (1)	O (1)
		XI (1)	E (1)
		XII (1)	n/a (1)

n/a, not available.

parts of Thailand such as Phitsanulok, lower northern (Niumsup *et al*, 2009); Bangkok, the capital city (Thapa *et al*, 2010); and Phetchaburi, western Thailand (Santimaleeworagun *et al*, 2014). However, this is the first report (to the best of our knowledge) of bla_{VIM2} identified in CRAB isolates in Thailand. In addition 41% of CRAB isolates carried more than one carbapenemase gene. Karthikeyan *et al* (2010) reported the co-existence of bla_{OXA} + bla_{NDM1} in three CRAB isolates from Indian patients. On the other hand, we detected bla_{OXA} + bla_{NDM1} and bla_{OXA} + bla_{VIM}. In the present study a new triple combination, bla_{OXA23} + bla_{VIM2} + bla_{NDM1}, was discovered, whereas the previous combination, bla_{OXA23} + bla_{IMP} + bla_{NDM}, is not unique (Chen *et al*, 2011a).

To date, there is no report as to how the presence of multiple types of carbapenemase genes came to be present in CRAB. We surmise that the resistance genes might be spread via clonal expansion or horizontal gene transfer. Previous studies showed that bla_{OXA23}, bla_{VIM}, and bla_{NDM} is inserted in mobile genetic element in *A. baumannii*, plasmid-borne integron in *P. aeruginosa* and plasmid in Enterobacteriaceae, respectively (Pallecchi *et al*, 2001; Yum *et al*, 2002; Yatsuyanagi *et al*, 2004; Lee *et al*, 2005; Thapa *et al*, 2010; Poirel *et al*, 2011). From REP patterns and plasmid profiles in this study, it is possible that bla_{OXA23} was spread clonally, and that bla_{VIM2} and bla_{NDM1} were transferred via plasmids. Plasmid profiling also revealed that certain plasmid types

were quite specific to certain resistance genes. For instance, plasmid profile A was responsible for CRAB harboring only bla_{OXA23}, whereas plasmid profiles B and C were specific for co-existence of different carbapenemase genes. Noticeably, one plasmid fragment was similar between plasmid profile B and C. It is possible that this plasmid might play a key role in the spread of co-existing carbapenemase genes.

A number of studies noted that the minimum inhibitory concentration (MIC) of imipenem against *A. baumanni* carrying bla_{OXA23} ranged from 16-256 μg/ml, and that the co-existence of different types of carbapenemases in *A. baumanii* increased MIC of imipenem to > 512 μg/ml (Poirel and Nordmann, 2006; Feizabadi *et al*, 2008; Karthikeyan *et al*, 2010). The effect on MIC of imipenem in CRAB harboring multiple carbapenemase gene types found in our study warrants further investigation.

In conclusions, our findings highlight the discovery of bla_{VIM2} and bla_{NDM1} and the co-existence of different carbapenemase genes in CRAB isolated from Thai patients. These findings should be taken into consideration as a serious health carbapenemase genes problem, which needs to be addressed. Furthermore, the presence of the various types of carbapenemase genes in CRAB and their epidemiology should be continuously monitored.

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REFERENCES

Chen Z, Qlu S, Wang Y, *et al*. Coexistence of bla_{NDM1} with the prevalent bla_{OXA23} and bla_{IMP} in pan-drug resistant *Acinetobacter baumannii* isolates in China. *Clin Infect Dis* 2011a; 52: 692-3.

Chen Y, Zhou Z, Jiang Y, Yu Y. Emergence of NDM-1-producing *Acinetobacter baumannii* in China. *J Antimicrob Chemother* 2011b; 66: 1255-9.

Clinical and Laboratory Standard Institute (CLSI). Performance standards for antimicrobial susceptibility testing: twenty-fifth informational supplement. Wayne: CLSI, 2015.

Durante-Mangoni E, Zarrilli R. Global spread of drug-resistant *Acinetobacter baumannii*: molecular epidemiology and management of antimicrobial resistance. *Future Microbiol* 2011; 6: 407-22.

Feizabadi MM, Fathollahzadeh B, Taherikalani M, *et al*. Antimicrobial susceptibility patterns and distribution of bla_{OXA} genes among *Acinetobacter* spp. isolated from patients at Tehran hospitals. *Jpn J Infect Dis* 2008; 61: 274-8.

Higgins PG, Dammhayn C, Hackel M, Seifert H. Global spread of carbapenem-resistant *Acinetobacter baumannii*. *J Antimicrob Chemother* 2010; 65: 233-8.

Johnson AP, Woodford N. Global spread of antibiotic resistance: the example of New Delhi metallo-beta-lactamase (NDM)-mediated carbapenem resistance. *J Med Microbiol* 2013; 62: 499-513.

Karthikeyan K, Thirunarayan MA, Krishnan P. Coexistence of bla_{OXA-23} with bla_{NDM-1} and *armA* in clinical isolates of *Acinetobacter baumannii* from India. *J Antimicrob Chemother* 2010; 65: 2253-4.

Lee K, Yong D, Yum JH, *et al*. Evaluation of Etest MBL for detection of bla_{IMP-1} and bla_{VIM-2} allele-positive clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J Clin Microbiol* 2005; 43: 942-4.

Mendes RE, Bell JM, Turnidge JD, Castanheira M, Jones RN. Emergence and widespread dissemination of OXA-23, -24/40 and -58 carbapenemases among *Acinetobacter* spp. in Asia-Pacific nations: report from the

SENTRY Surveillance Program. *J Antimicrob Chemother* 2009; 63: 55-9.

National Antimicrobial Resistance Surveillance Center (NARST). Antimicrobial resistance 2000-2015. Nonthaburi: National Antimicrobial Resistance Surveillance Center, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. [Cited 2016 Mar 10]. Available from: http://narst.dmsc.moph.go.th/data/AMR2000-2015.pdf

Niumsup PR, Boonkerd N, Tansawai U, Tiloklurs M. Carbapenem-resistant *Acinetobacter baumannii* producing OXA-23 in Thailand. *Jpn J Infect Dis* 2009; 62: 152-4.

Pallecchi L, Riccio ML, Docquier JD, Fontana R, Rossolini GM. Molecular heterogeneity of bla_{VIM-2}-containing integrons from *Pseudomonas aeruginosa* plasmids encoding the VIM-2 metallo-beta-lactamase. *FEMS Microbiol Lett* 2001; 195: 145-50.

Poirel L, Dortet L, Bernabeu S, Nordmann P. Genetic features of bla_{NDM-1}-positive enterobacteriaceae. *Antimicrob Agents Chemother* 2011; 55: 5403-7.

Poirel L, Nordmann P. Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clin Microbiol Infect* 2006; 12: 826-36.

Santimaleeworagun W, Thathong A, Samret W, Preechachuawong P, Sae-lim W, Jitwasinkul T. Identification and characterization of carbapenemase genes in clinical isolates of carbapenem-resistant *Acinetobacter baumannii* from general hospital in Thailand. *Southeast Asian J Trop Med Public Health* 2014; 45: 874-80.

Thapa B, Tribuddharat C, Srifuengfung S, Dhiraputra C. High prevalence of bla_{OXA-23} in oligoclonal carbapenem-resistant *Acinetobacter baumannii* from Siriraj Hospital, Mahidol University, Bangkok, Thailand. *Southeast Asian J Trop Med Public Health* 2010; 41: 625-35.

Tsakris A, Ikonomidis A, Pournaras S, *et al*. VIM-1 metallo-beta-lactamase in *Acinetobacter baumannii*. *Emerg Infect Dis* 2006; 12: 981-3.

Walsh TR, Weeks J, Livermore DM, Toleman MA. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect Dis* 2011; 11: 355-62.

Yatsuyanagi J, Saito S, Harata S, *et al*. Class 1 integron containing metallo-beta-lactamase gene bla_{VIM-2} in *Pseudomonas aeruginosa* clinical strains isolated in Japan. *Antimicrob Agents Chemother* 2004; 48: 626-8.

Yum JH, Yi K, Lee H, *et al*. Molecular characterization of metallo-beta-lactamase-producing *Acinetobacter baumannii* and *Acinetobacter* genomospecies 3 from Korea: identification of two new integrons carrying the bla_{VIM-2} gene cassettes. *J Antimicrob Chemother* 2002; 49: 837-40.