BLA$_{\text{OXA-23-LIKE}}$ PRESENCE AND 29 KDA PORIN LOSS IN CARBAPENEM RESISTANT *ACINETOBACTER BAUMANNII* CLINICAL ISOLATES IN THAILAND

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Abstract. In Thailand, the incidence of carbapenem resistant *Acinetobacter baumannii* (CRAB) is dramatically increasing. OXA-23 carbapenemase has been identified in clinical isolates; however, other mechanisms remain unclear. PCR was used to amplify OXA, MBL and ade genes. Outer membrane protein (OMP) components were analyzed by SDS-PAGE and carbonyl cyanide m-chlorophenyl hydrazone was employed to detect the role of efflux pumps. Of 22 clinical CRAB isolates from sterile sites, 16 carried *bla*$_{\text{OXA-23}}$ and the remaining *bla*$_{\text{OXA-23-like}}$; all were negative for the presence of other OXA and MBL gene types. AdeJ, but inactive, efflux pump system was found in all 8 randomly selected CRAB isolates and a 29 kDa OMP was absent. This is the first such properties of clinical CRAB isolates in Thailand and their roles in contributing to *A. baumannii* carbapenem resistance need further investigation.

Keywords: *Acinetobacter baumannii*, *bla*$_{\text{OXA-23}}$, outer membrane protein, porin

INTRODUCTION

Acinetobacter baumanni, a gram-negative coccobacillus, causes serious nosocomial infections such as pneumonia, bacteremia and skin/soft tissue infection including a rare case with meningitis (Howard *et al*, 2012). In addition, *A. baumannii* infection is correlated with increased hospital stay and cost, and mortality (Lee *et al*, 2007; Esterly *et al*, 2011).

Carbapenems frequently are used to treat *A. baumannii* (Doi *et al*, 2015); however, increasing incidence of carbapenem-resistant *A. baumannii* (CRAB) has been noted (NARST, 2016). The mechanisms underlying CRAB include enzymatic inactivation, loss of porins, cellular export via efflux pumps, and changes in

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properties of penicillin-binding proteins (PBPs) (Lin and Lan, 2014). Among these various mechanisms, cleavage of β-lactam ring by carbapenemases, such as OXA-carbapenemases and metallo-β-lactamase (MBL) is most commonly reported (Lin and Lan, 2014; Doi *et al*, 2015).

Resistance-nodulation cell division (RND) family of efflux pumps, whose members include adeABC, adeFGH and adeIJK, causes resistance to multiple classes of antimicrobials in *A. baumannii* (Lin and Lan, 2014). These efflux pumps not only produce resistance to aminoglycoside, chloramphenicol, erythromycin, fluoroquinolones, tetracyclines, and trimethoprim (Li and Nikaido, 2004) but also to tigecycline (Hornsey *et al*, 2010). Reduced expression of porins of 22, 29 (CarO protein), 33-36, 37, 44, and 47 kDa located in outer membrane of CRAB also were documented (Lin and Lan, 2014).

In Thailand, $bla_{\text{OXA-23}}$ is the OXA-type most often found in CRAB (Niumsup *et al*, 2009; Thapa *et al*, 2010; Santimaleeworagun *et al*, 2011), but the $bla_{\text{OXA-40}}$ was reported (Santimaleeworagun *et al*, 2014). In the present study, we examined bla_{OXA} and bla_{MBL}, role of efflux pumps and status of porin in CRAB isolates obtained from sterile sites of hospitalized patients.

MATERIALS AND METHODS

Bacterial strains

Clinical isolates were gathered from patients admitted to Songklanagarind Hospital, an affiliate medical school of Prince of Songkla University, southern Thailand during January-December 2008. Inclusion criteria were ability to obtain CRAB isolates from sterile sites [*eg*, blood, cerebrospinal fluid (CSF), bone marrow, peritoneal fluid, pleural fluid, or synovial fluid]. All strains were kept at -20°C until used.

The study was approved by the ethics committee of the Faculty of Medicine, Prince of Songkla University (permit no. SUB.EC 52-030-19-2-3).

Determination of antimicrobial susceptibility

Antimicrobial susceptibility against β-lactam antibiotics [ceftazidime (30 μg), meropenem (10 μg), and piperacillin/tazobactam (100 μg/10 μg)], aminoglycosides [amikacin (30 μg) and gentamicin (10 μg)], ciprofloxacin (5 μg), and trimethoprim/sulfamethoxazole (1.25 μg/23.75 μg) were evaluated using a disk diffusion method on agar plate (CLSI, 2016). Susceptibilities to colistin (0.13-4 μg/ml), β-lactam imipenem (0.03-128 μg/ml), and sulbactam (2-128 μg/ml) were determined by an agar dilution method according to CLSI (2016) protocol. *Escherichia coli* ATCC 25922 was used as quality control.

PCR amplification of OXA, MBL and ade genes

Genomic DNA was extracted from bacterial cell using DNA extraction commercial kit (Qiagen, Valencia, CA) and employed as template to PCR amplify $bla_{\text{IMP-like}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-40}}$, $bla_{\text{OXA-58}}$, $bla_{\text{SIM-1}}$, $bla_{\text{VIM-1}}$, $bla_{\text{VIM-2}}$, IS*Aba1* elements upstream of the bla_{OXA} genes, and genes of adeB, adeJ and adeY [main transporter proteins (MTPs)] using target-specific primer pairs (Table 1). Reactions were carried out in 50-μl mixture containing 1 μl of genomic DNA, 10 mM each primer pair, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 5 μl of 10X PCR buffer and 1.25 U *Taq* (Invitrogen, Carlsbad, CA). Thermocycling was performed in Takara gradient instrument (Tokyo, Japan) as follows: 30 cycles of 94°C for 60 seconds; followed by the appropriate temperature for target gene (Table 1) for

Table 1
Primers used in the study.

Gene	Primer sequence	Amplicon size (bp)	GenBank accession number
bla$_{IMP\text{-like}}$[1]	F- 5`CTRCCGCAGSAGMGBCTTTG3` R- 5`AACCAGTTTTGCHTTACCAT3`	587	AJ640197 AJ243491 AY590475 AF290912 EF127959 AB074436 AB184977
bla$_{OXA\text{-}23}$	F- 5`GGAATTCCATGAATAAATATTTTA3` R- 5`GGATCCCGTTAAATAATATTCAGC3`	822	AJ132105
bla$_{OXA\text{-}24}$	F-5`GGAATTCCGTACTAATCAAAGTTGTGAA3` R- 5`GGATCCCGTTCCCCTAACATGAATTTGT3`	828	AJ239129
bla$_{OXA\text{-}40}$	F- 5`GGAATTCCATGAAAAAATTTATAC3` R- 5`GGATCCCGTTAAATGATTCCAAGA3`	828	AF509241
bla$_{OXA\text{-}58}$	F- 5`GGAATTCCATGAAATTATTAAAAA3` R- 5`GGATCCCGTTATAAATAATGAAAA3`	843	EU107365
bla$_{SIM\text{-}1}$	F- 5`GGAATTCCATGAGAACTTTATTGA3` R- 5`GGATCCCGTTAATTAATGAGCGGC3`	741	AY887066
bla$_{VIM\text{-}1}$	F- 5`GGAATTCCATGTTAAAAGTTATTA3` R- 5`GGATCCCGCTACTCGGCGACTGAG3`	801	DQ112355
bla$_{VIM\text{-}2}$	F- 5`GGAATTCCATGTTCAAACTTTTGA3` R- 5`GGATCCCGCTACTCAACGACTGAG3`	801	AF291420
IS*Aba1*	F- 5`CACGAATGCAGAAGTTG3` R- 5`CGACGAATACTATGACAC3`	563	GQ849192
Ade[2]	F- 5`ATGKCACAATTTTTTATTC3` R- 5`CACAAAGTGCCGGTGT3`	1,491	AF370885 AY769962 DQ223769

[1]Degenerate primer pair to amplify IMP-1, IMP-2, IMP-4, IM-5, IMP-8, IMP-11 and IMP-19 gene fragments; [2]Degenerate primer pair to amply AdeB, Ade J and AdeY gene fragments. B = C, G or T; H = A, C or T; K = G or T; M = A or C; R = A or G; S = C or G.

60 seconds and then 72ºC for 120 seconds. Amplicons were analyzed by 1% agarose gel-electrophoresis, inserted into Strataclone PCR cloning kit (Stratagene, La Jolla, CA) and transfected into One Shot™ TOP10 Chemically Competent *E. coli* (Invitrogen). Recombinant plasmids were extracted using Illustra plasmid Prep Mini Spin Kit (GE Health Care, Little Chalfont, UK) and inserts sequenced (Ward Medic, Bangkok, Thailand). Sequences were compared with those present in GenBank and novel sequences deposited as accession nos. HM488986-HM488992.

Detection of the efflux pump activity

CRAB isolates were grown on Mueller Hinton agar (Beckton Dickinson, Baltimore, MD) with and without two-fold dilutions of 100 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP; Sigma-Aldrich, St Louis, MO), an efflux pump

inhibitor (Huang *et al*, 2008). Significant decrease in MIC of imipenem after more than four two-fold dilutions of CCCP compared with MIC of imipenem alone was considered positive for efflux activity. Each experiment was conducted in duplicate.

Characterization of CRAB outer membrane proteins (OMPs)

Eight randomly selected CRAB strains were classified into two groups: four each with imipenem MIC = 16 mg/l and ≥ 32 mg/l. CSAB (carbapenem sensitive *A. baumannii*) strain AB19, isolated from blood, imipenem MIC = 1 mg/l, was used as control. OMPs were isolated from cells at the logarithmic phase of growth as previously described (Jyothisri *et al*, 1999). In brief, each strain was grown in 300 ml of LB broth (Beckton Dickinson) until 0.6 $OD_{600\ nm}$ and lysed by sonication. Cells were centrifuged at 4,000*g* for 5 minutes at 4°C and then supernatant was centrifuged at 100,000*g* at 4°C for 60 minutes. The bacterial pellet was suspended in 2% N-lauryl sarcosine sodium and centrifuged at 100,000*g* for 60 minutes at 4°C. OMP fraction (pellet) was solubilized in 20 mM Tris-HCl pH 8.0 containing 0.1mM ethylene diaminetetraacetic acid and 1% SDS and stored at -20°C until analyzed. OMP (20 μg) was heated at 95°C for 5 minutes and analyzed by 4-20% gradient SDS-PAGE followed by staining with Coomassie brilliant blue dye. Protein concentration was determined using Bradford's method.

RESULTS

Source and antimicrobial susceptibility of CRAB isolates

Among 110 clinical *A. baumannii* isolates from patients' sterile sites, 22 CRAB isolates were obtained, 14 (64%) from blood, 3 (14%) from intraabdominal tissues, 2 (9%) from CSF, 2 (9%) from ascites fluid, and 1 (4%) from other site. Using disk or agar dilution method, all CRAB isolates were resistant to ampicillin, cefepime, ceftazidime, ciprofloxacin, colistin, imipenem, meropenem and piperacillin/tazobactam, followed by 86% to cefoperazone/sulbactam, 27% to amikacin, 23% to gentamicin, and 9% to trimethoprim/sulfamethoxazole (Table 2). The MIC range of colistin was 0.5-1 mg/l, imipenem 16-64 mg/l and sulbactam 8-64 mg/l.

OXA and MBL carbapenemase genes in CRAB isolates

PCR amplification of the 22 CRAB isolates from patients' sterile sites showed all isolates carried $bla_{\text{OXA-23-likes}}$ but not $bla_{\text{OXA-40}}$, $bla_{\text{OXA-58}}$, $bla_{\text{IMP-likes}}$, $bla_{\text{SIM-1}}$, $bla_{\text{VIM-1}}$ and $bla_{\text{VIM-2}}$. Among the 22 strains producing OXA-23-like carbapenemase, sequencing analysis revealed six isolates harboring new variant $bla_{\text{OXA-23}}$, namely, $bla_{\text{OXA-165}}$ (I223V), $bla_{\text{OXA-166}}$ (V52A), $bla_{\text{OXA-167}}$ (L263M), $bla_{\text{OXA-169}}$ (K47R), $bla_{\text{OXA-170}}$ (Q207K), and $bla_{\text{OXA-171}}$ (I258T). The IS*Aba1* element was found upstream of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-23-likes}}$.

Efflux pump in CRAB isolates

PCR amplification showed the presence of a 1,491-bp ade gene fragment in eight randomly chosen CRAB isolates (data not shown) and subsequent sequencing demonstrated that it encodes AdeIJK of the RND superfamily. However, CCCP inhibition assay showed the pump was not functioning as demonstrated by the absence of significant changes in imipenem MIC with and without CCCP (Table 2).

OMPs

SDS-PAGE analysis of the eight randomly chosen CRAB isolates revealed an

Table 2
Characteristics of twenty-two carbapenem-resistant *Acinetobacter baumannii* clinical isolates and minimum inhibitory concentrations (MICs) against antimicrobial agents.

Strain ID	Specimen source	MIC (mg/l)			
		IMP	IPM+CCCP	COL	SUL
AB11	Blood	32	16	1	8
AB13	Ascitic fluid	32	32	1	8
AB15	Pancrease tissue	32	32	1	8
AB23	Blood	32	32	1	8
AB54	Intra-abdominal tissue	16	16	1	8
AB58	Intra-abdominal tissue	16	16	1	32
AB77	Blood	32	16	1	64
AB79	Blood	64	32	1	32
AB133	Blood	16	16	1	32
AB153	Blood	16	16	1	16
AB164	Blood	16	16	1	32
AB167	Blood	32	16	1	32
AB179	Blood	16	16	1	32
AB198	CSF[2]	32	16	1	32
AB271	Other	16	16	1	32
AB272	Blood	16	16	1	16
AB286	Blood	16	16	1	16
AB307	Blood	64	64	1	64
AB313	Ascitic fluid	16	16	1	16
AB315	Blood	16	16	0.5	64
AB316	Blood	32	16	1	32
AB322	Cerebrospinal fluid	32	32	1	16

COL, colistin; IMP, imipenem; IMP+CCCP, imipenem + carbonyl cyanide m-chlorophenyl hydrazine; SUL, sulbactam.

absence of 29 kDa OMP but no other differences when compared to CSAB strain (AB19) (Fig 1).

DISCUSSION

Nosocomial infections due to CRAB is increasing worldwide (Lin and Lan, 2014) including Thailand (NARST, 2016). Treatment of CRAB infection is challenging due to multi-mechanisms of drug resistance (Doi *et al*, 2015). Only bla_{OXA23} has been previously reported (Niumsup *et al*, 2009; Thapa *et al*, 2010; Santimaleeworagun *et al*, 2011; Santimaleeworagun *et al*, 2014) but not bla_{IMP} (Niumsup *et al*, 2009) in CRAB isolates in Thailand in keeping with our findings, which identified a number of novel OXA-23 variants.

While carbapenemases are the predominant causes for CRAB, efflux pump and loss of porins have also been shown to be responsible. Magnet *et al* (2001) isolated MDR-*A. baumannii* strain BM4454 carrying *adeb* of the RND system. Lin *et al* (2009) found AdeJ in *A. baumannii*-*A.*

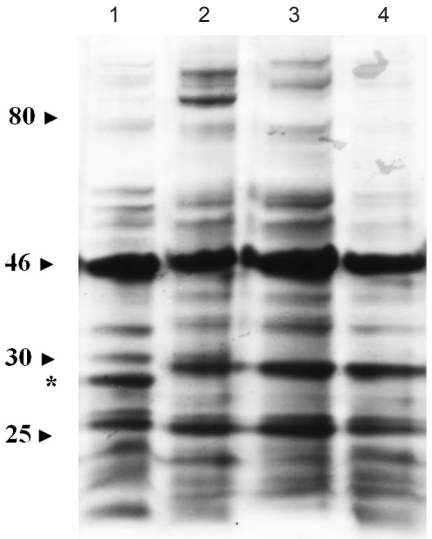

Fig 1–SDS-PAGE separation of outer membrane proteins from carbapenem-resistant *Acinetobacter baumannii* clinical isolates. Cultured cells were subjected to sonication, centrifuged and pellet was separated by 4-20% SDS-PAGE followed by staining with Coomassie brilliant blue. Lane 1, carbapenem susceptible *A. baumannii*; lanes 2-4, AB23, AB54 and AB164, respectively (from Table 2). Molecular weight markers (kDa) are indicated in left margin. *29 kDa.

calcoaceticus complex isolated from hospitalized patients in Sichuan, China. Our finding of AdeIJK gene expression is, to the best of our knowledge, the first report of its kind in CRAB from Thailand. It is worth noting that the efflux pump was inactive for imipenem. As the RND system is the most common in *A. baumannii*, we did not attempt to examine the existence of other efflux pump systems (*eg*, MATE and MFS families) found in *A. baumannii* (Vila *et al*, 2007).

As previously reported, carbapenem-resistant bacterial isolates have reduced expression of porins of various sizes (Lin and Lan, 2014). The loss of 29 kDa OMP detected in our CRAB strains is similar to the report of Limansky *et al* (2002). However, the functional significance of this property needs to be further investigated.

There were certain limitations to our study: 1) only the most common mechanisms in CRAB were investigated, 2) the presence of OMP and efflux pump were studied in a limited number of CRAB isolates, 3) the ability of the six OXA-23 variants to cleave β-lactam antimicrobial agents and their kinetic properties were not examined; motifs S-T-F-$K_{69\text{-}72}$, F-G-$N_{152\text{-}154}$ and K-T-$G_{216\text{-}218}$, located on the enzyme surface do not affect activity (Donald *et al*, 2000; Brown and Amyes, 2006).

In summary, this is the first report of CRAB isolate from Thailand carrying $bla_{\text{OXA-23-likes}}$, loss of 29-kDa porin and an inactive (imipenem) efflux pump. The contributions of these findings need further detailed investigations.

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CONFLICTS OF INTEREST

The author declare no conflicts of interest.

REFERENCES

Brown S, Amyes S. OXA (beta)-lactamases in Acinetobacter: the story so far. *J Antimicrob Chemother* 2006; 57: 1-3.

Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; twenty-sixth informational supplement. Wayne: CLSI, 2016.

Doi Y, Murray GL, Peleg AY. *Acinetobacter baumannii*: evolution of antimicrobial resistance-treatment options. *Semin Respir Crit Care Med* 2015; 36: 85-98.

Donald HM, Scaife W, Amyes SG, Young HK. Sequence analysis of ARI-1, a novel OXA beta-lactamase, responsible for imipenem resistance in *Acinetobacter baumannii* 6B92. *Antimicrob Agents Chemother* 2000; 44: 196-9.

Esterly JS, Griffith M, Qi C, Malczynski M, Postelnick MJ, Scheetz MH. Impact of carbapenem resistance and receipt of active antimicrobial therapy on clinical outcomes of *Acinetobacter baumannii* bloodstream infections. *Antimicrob Agents Chemother* 2011; 55: 4844-9.

Hornsey M, Ellington MJ, Doumith M, *et al*. AdeABC-mediated efflux and tigecycline MICs for epidemic clones of *Acinetobacter baumannii*. *J Antimicrob Chemother* 2010; 65: 1589-93.

Howard A, O'Donoghue M, Feeney A, Sleator RD. *Acinetobacter baumannii*: an emerging opportunistic pathogen. *Virulence* 2012; 3: 243-50.

Huang L, Sun L, Xu G, Xia T. Differential susceptibility to carbapenems due to the AdeABC efflux pump among nosocomial outbreak isolates of *Acinetobacter baumannii* in a Chinese hospital. *Diagn Microbiol Infect Dis* 2008; 62: 326-32.

Jyothisri K, Deepak V, Rajeswari MR. Purification and characterization of a major 40 kDa outer membrane protein of *Acinetobacter baumannii*. *FEBS Lett* 1999; 443: 57-60.

Lee NY, Lee HC, Ko NY, *et al*. Clinical and economic impact of multidrug resistance in nosocomial *Acinetobacter baumannii* bacteremia. *Infect Control Hosp Epidemiol* 2007; 28**:** 713-9.

Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria. *Drugs* 2004; 64: 159-204.

Limansky AS, Mussi MA, Viale AM. Loss of a 29-kilodalton outer membrane protein in *Acinetobacter baumannii* is associated with imipenem resistance. *J Clin Microbiol* 2002; 40: 4776-8.

Lin L, Ling BD, Li XZ. Distribution of the multidrug efflux pump genes, *adeABC, adeDE* and *adeIJK*, and class 1 integron genes in multiple-antimicrobial-resistant clinical isolates of *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex. *Int J Antimicrob Agents* 2009; 33: 27-32.

Lin MF, Lan CY. Antimicrobial resistance in *Acinetobacter baumannii*: from bench to bedside. *World J Clin Cases* 2014; 2: 787-814.

Magnet S, Courvalin P, Lambert T. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrob Agents Chemother* 2001; 45: 3375-80.

National Antimicrobial Resistance Surveillance Center, Thailand. (NARST). National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. Antibiogram 2016. Nonthaburi: NARST, 2016. [Cited 2017 May 10]. Available from: http://narst.dmsc.moph.go.th/

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.

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.

Santimaleeworagun W, Wongpoowarak P, Chayakul P, Pattharachayakul S, Tansakul P, Garey KW. *In vitro* activity of colistin or sulbactam in combination with fosfomycin or imipenem against clinical isolates of carbapenem-resistant *Acinetobacter baumannii* producing OXA-23 carbapenemases. *Southeast Asian J Trop Med Public Health* 2011; 42: 890-900.

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.

Vila J, Marti S, Sanchez-Cespedes J. Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. *J Antimicrob Chemother* 2007; 59: 1210-5.