Research report

Identification of carbapenemase genes in *Acinetobacter baumannii* clinical isolates from Songklanagarind hospital

การตรวจหายีนคาร์บาพีเนมเมสในเชื้อ *Acinetobacter baumannii* ที่แยกได้จากผู้ป่วยในโรงพยาบาลสงขลานครินทร์

Payom Wongpoowarak

Pimpimon Tansakul

Sutthiporn Pattharachayakul

Department of Clinical Pharmacy

Faculty of Pharmaceutical Sciences

Prince of Songkla University

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Primary investigator: Payom Wongpoowarak, Ph.D.

Department of Clinical Pharmacy

Faculty of Pharmaceutical Sciences

Prince of Songkla University

Hat Yai, Songkhla 90112, THAILAND

Email:payom.w@psu.ac.th

Co-worker: Pimpimon Tansakul, Ph.D.

Department of Pharmacognosy and Pharmaceutical Botany,

Email: pimpimon.t@psu.ac.th

Sutthiporn Pattharachayakul

Department of Clinical Pharmacy

Email: sutthiporn.p@psu.ac.th

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Abstract

At the present, the incidence of carbapenem resistant *Acinetobacter baumannii* (CRAB) is dramatically increasing in Thailand. Because of multi-mechanisms of drug resistance in CRAB, thus treatment with antimicrobial is difficult which lead to high morbidity and mortality. In this study, we aim to identify types of IMP and OXA resistant genes in carbapenem-resistant *Acinetobacter baumannii* clinical isolates by polymerase chain reaction technique. The CRAB clinical isolates from sterile sites in patients admitted to Songklanagarind hospital during January-December 2008, were studied. All of the isolates (n=22) carried the $bla_{OXA\text{-}23\text{-likes}}$ genes; sixteen isolates carried $bla_{OXA\text{-}23}$ genes and other 6 isolates harboring new variant $bla_{OXA\text{-}23\text{-likes}}$ genes. In conclusion, this study reported OXA-23 and OXA-23 like carbapenemases that were majority of carbapenem resistance. These new variant types have to be studied in enzyme activity

Keywords : *Acinetobacter baumannii*, carbapenemase, oxacilinase, imipenemase

บทคัดย่อ

ในปัจจุบันอุบัติการณ์ของ *Acinetobacter baumannii* ที่ดื้อยากลุ่มคาร์บาพีเนมเพิ่มสูงขึ้นอย่างต่อเนื่องในประเทศไทย เนื่องจากเชื้อมีหลายกลไกในการดื้อยา การรักษาด้วยยาต้านจุลชีพจึงมีความยุ่งยากซับซ้อน อีกทั้งโรคติดเชื้อที่มีสาเหตุจาก *A. baumannii* ก่อให้เกิดทุพพลภาพและเสียชีวิตได้สูง การวิจัยนี้เป็นส่วนหนึ่งของการศึกษาเพื่อพิสูจน์ยีนดื้อยากลุ่มคาร์บาพีเนม โดยในการวิจัยนี้มีวัตถุประสงค์เพื่อระบุชนิดของยีน IMP และ/หรือ OXA ซึ่งเป็นยีนดื้อยากลุ่ม คาร์บาพีเนม โดยใช้เทคนิค polymerase chain reaction ในสิ่งส่งตรวจที่ไม่มีการปนเปื้อนซึ่งแยกได้จากผู้ป่วยที่นอนรักษาตัวในโรงพยาบาลสงขลานครินทร์ ระหว่างเดือน มกราคม-ธันวาคม พ.ศ. 2551

เชื้อดื้อยากลุ่มคาร์บาพีเนม ทั้ง 22 สายพันธุ์ตรวจพบยีน $bla_{OXA\text{-}23\text{-likes}}$ โดย 16 สายพันธุ์เป็นยีน $bla_{OXA\text{-}23}$ และ 6 สายพันธุ์เป็นยีนชนิดใหม่ในกลุ่ม $bla_{OXA\text{-}23\text{-likes}}$ โดยสรุปการศึกษานี้รายงานการดื้อยากลุ่มคาร์บาพีเนมด้วยกลไกสร้างเอนไซม์ OXA-23 และเป็นยีนชนิดใหม่ในกลุ่ม $bla_{OXA\text{-}23\text{-likes}}$ ซึ่งต้องมีการการศึกษาวิจัยต่อไปเกี่ยวกับสมรรถนะของเอนไซม์ชนิดใหม่ดังกล่าว

คำสำคัญ: อะซีเนโตแบคเตอร์ บาวมานนิอิ, คาร์บาพีเนมเมส. ออกซาซิลลิเนส. อิมิพีเนมเมส

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Lastly, I offer my regards and blessings to all of my patients who gave me the valuable data for doing project. Your information will help and give a good chance for other patients. With your goodness, it will turn back to you to have a good times, good health and peaceful.

Content

1. Introduction

1.1. Rationale

Acinetobacter baumanni is gram-negative bacilli which causes nosocomial infections worldwide. A large surveillance study examined 24,179 cases of nosocomial bloodstream infections (BSI) from March 1995 through September 2002 in the United States. The findings indicated that *Acinetobacter* species was ranked the second in causes of mortality (43.4%) in patients admitted to intensive care units (Wisplinghoff et al., 2004). In Spain, the national survey of acquired invasive device-related infections found that *A. baumannii* was the third rank of isolated pathogen in patients with mechanical ventilator occurring 8.3 and 8.8 % in 2003 and 2005 respectively (Alvarez-Lerma et al., 2007). Like the increasing rate of *A. bamannii* infection in the western countries, the incidence of nosocomial infection due to *A. baumannii* in Taiwan were increasing 25 to 55 episodes per 10,000 discharged patients between 1999 and 2003 (Hsueh, Chen, & Luh, 2005). Similar to the other countries, the data of National Antimicrobial Resistance Surveillance Center Thailand (NARST) reported that *A. bauamannii* was identified in patients sputum in the years 2004, 2005 and 2006 at a rate 14, 14, and 17% of isolated organisms showed that, respectively. In addition, the isolated organisms from all specimens in patients during 2005-2007, *A. baumannii* was presented in 6-7% and rank fourth of isolated clinical pathogens (NARST, 2007).

A. baumannii related infections are associated with severe adverse outcomes, including increased mortality, prolonged hospitalization, additional hospital costs, and reduced functional status. Lee et al. found that the bacteremia due to multi-drug resistant *A. baumannii* (MDR-*A. baumannii*) resulted in 13.4 days of additional hospitalization and US$ 3,758 of additional costs (Lee et al., 2007). Another study showed that patients with MDR-*A. baumannii* infection in a surgical intensive care unit had a mean of $60,913 in attributable excess patient charges and a mean of 13 excess hospital days (Young, Sabel, & Price, 2007). Moreover, evidence showed that MDR-*A. baumannii* lead to a high mortality rate. Kuo et al. found that the overall 30-day mortality rate was 49% (Kuo et al., 2007). In addition, the study of Tseng et al. also reported the crude mortality rates in patients infected with carbapenem-resistant *A. baumannii* (CR-*A. baumannii*) on days 2,

7, 15, 30, and at discharge were 23.2%, 30.4%, 37.5%, 48.2%, and 60.7%, respectively (Tseng et al., 2007).

The important causes of *A. baumannii* infection that result in high morbidity and mortality has been hypothesized to be due to three properties of this pathogen which includes the cell structure, persistence in environment, and multi-mechanism of drug resistance.

The cell structure express the high virulence consisting of four characteristics; (1) the presence of a polysaccharide capsule, (2) the property of adhesion to human epithelial cells, (3) the production of enzymes which may damage tissue lipids and (4) the potentially toxic role of the lipopolysaccharide component of the cell wall. The second reason for *A. bauamannii* infections was the property of prolonged environmental contamination. The epidemiological evidences were reported for up to 13 days after the discharge of a patient and the dissemination of *Acinetobacter* spp. was seen in an outbreak over a 21-month period (Bergogne-Berezin & Towner, 1996). Finally, the majority *A. baumannii* resistance is due to multiple mechanisms.

In the present, the problems of resistant *A. baumannii* are dramatically increasing. Several studies have documented the multi-mechanism of resistant *A. baumannii* via 1) enzymatic lyses (including oxacilinases (OXA), metalo-β-lactamases (MBL), Amp-C, Aminoglycoside-modifying enzyme), 2) losing of porins, 3) efflux pump, or 4) changing of receptor of drug (penicillin-binding proteins (PBPs), ribosomal RNA, or DNA gyrase). Particularly, carbapenemases are the enzyme group having activity in cleavage of the β-lactame ring of carbapenems, cephalosporins and penicillins. The carbapenemases have been categorized into two major subtypes due to molecular structure and the essential co-factors to activate the reaction including OXA-carbapenemases and MBL (Bergogne-Berezin & Towner, 1996).

OXA-carbapenemases have been classified by molecular structure into group D β-lactamases or by functional or substrate group into class 2d. Currently, there are eight subgroups in OXA-type carbapenemases and only four of them have been verified in *A. baumannii*. The first subgroup includes OXA-23, OXA-27, and OXA-49. The second subgroup consists of four enzymes in this subgroup including OXA-24, -25, -26, and -40. The third group has the OXA-51 family which is intrinsically found in *A. baumannii* whereas only OXA-58 type in the fourth group (Poirel & Nordmann, 2006; Walther-Rasmussen & Hoiby, 2006). Many studies have examined the occurring of genes OXA-

carbapenemase. OXA-23 and OXA-51-like genes have been examined in Sydney, Australia (Valenzuela et al., 2007), OXA-40 occurring in the United States (Lolans, Rice, Munoz-Price, & Quinn, 2006) and, a blaOXA-51-like gene was amplified from clinical isolates in Naples, Italy (Zarrilli et al., 2007) and from pediatric patients in Greece (Poirel & Nordmann, 2006).

MBLs, like OXA-carbapenemase, can hydrolyze carbapenems and all other β-lactams. Although, MBLs need Zinc (Zn) for their reaction. At a molecular level, MBLs are classified by structure in group B or by functional or substrate group in class E of Bush-Jacoby Medeiros system. To date, three MBLs subtypes have been identified in *A. baumannii*, IMP-like (imipenemase), VIM-like (Verona-imipenemase), and SIM-1 (Seoul-imipenemase) (Poirel & Nordmann, 2006; Walsh, Toleman, Poirel, & Nordmann, 2005). In the epidemiologic studies of MBL production, Lim et al. reported that among 40 strains, a single isolate (2.5%) was an MBL producer carrying IMP-1 by PCR method, but VIM-type MBLs were not found (Lim, Shin, & Kim, 2007). On the contrary, the study of Wroblewska et al. found that MBLs were negative, but VIM-type MBLs were positive for CR-*A. baumannii* isolates belonging to patients in various wards of the hospital in Poland (Wroblewska, Rudnicka, Marchel, & Luczak, 2006).

Because of resistance and high virulence of *A. baumannii*, treatment of active drugs are difficult. In vitro studies with colistin and rifampin, sulbactam and imipenem, rifampicin and imipenem have shown the synergistic effects on MDR-*A. baumannii.* However, these synergies did not evolve in all resistant isolates. The synergism of any combination of antimicrobials depends on the resistant gene that was carried by the organism (Giamarellos-Bourboulis, Xirouchaki, & Giamarellou, 2001). Certain *A. baumannii* strains did not differ in their level of resistance to imipenem (estimated minimum inhibitory concentration ,MIC at 8-32 µg/mL) but the results of synergic effects of antimicrobial agents which combat them has been controversial. Tripodi et al. compared the activities of antibiotic combination against *A. baumannii* producing OXA-58 carbapenemase (imipenem resistance, MIC 16 ug/mL). Synergism was observed with combinations of colistin plus rifampicin or imipenem plus rifampicin in all of isolates (Tripodi, Durante-Mangoni, Fortunato, Utili, & Zarrilli, 2007). These findings disagree with the results of Montero et al's who performed combinations of antibiotic in encoding OXA-24 strain (imipenem resistance, MIC 8 ug/mL) (Montero et al., 2004). The addition of rifampicin to imipenem did not result in synergistic outcome.

Investigation of a new antimicrobial agent which contains activity against *A. baumannii* has been performed. Fosfomycin, which is a phosphonic acid derivative, has the antibacterial effects to inhibit of bacterial cell-wall synthesis. In vitro, fosfomycin has demonstrated activity against gram-positive bacteria e.g. *Staphylococcus spp*., *Streptococcus spp.* and *Entercoccus spp*. In addition, fosfomycin also has an activity against some gram negative bacteria including extended-spectrum beta-lactamase (ESBL)-producing microorganisms (de Cueto, Hernandez, Lopez-Cerero, Morillo, & Pascual, 2006). Since the fosfomycin in plasma has minimum inhibitory concentration (MIC) within therapeutic range, the possibility of using fosfomycin for MDR-*A. baumannii* infection has been evaluated. Falagas et al. found that MIC of fosfomycin was a range of 64 to over 512 µ/ml (Falagas et al., 2008). Moreover, the susceptibility data of fosfomycin in carbapenem resistant *A. baummnii* (CR-*A. baumannii*) have been studied by infectious diseases and tropical diseases unit, Songklanagarind hospital, the results indicated that fosfomycin might be effective agent. The MIC50 and MIC90 results for fosfomycin were 64 and 96 µg/mL, respectively. Fosfomycin may be a new antimicrobial agent for the treatment of infections due to MDR-*A. baumannii* strains.

As above-mentioned, the uses of combination of antimicrobial regimens need to be tested in individual settings because of the difference of the epidemiology of resistant genes in order to choose the efficacious antibiotic combination for treatment. In addition, the study of effective combinations against *A. baumannii* strains expressing different mechanisms of carbapenem resistance needs to be done.

Only two studies of resistant genes in MDR-*A. baumannii* have been done in Thailand. First, Boonkerd et al. detected β-lactamase genes in 16 clinical strains of MDR-*A. baumannii* isolated from patients admitted in Buddhachinaraj Hospital, Phitsanulok from February to April 2006. The results of the detection of carbapenem resistant genes showed that six isolates carried IMP and 15-*A. baumannii* isolates carried OXA-23 (Boonkerd, Pibalpakdi, Tiloklurs, & Niumsup, 2009) Conversely, the study of Duangseesai et al. determining the resistant genes in 30 strains of imipenem resistant-*A. Baumannii* at Chulalongkorn hospital, Bangkok showed OXA-23 in 36.67% of isolates but IMP-gene could not be detected in all isolates (Duangseesai, Fungwitthaya, & Pongpech, 2005). These results suggested that IMP and OXA-23 carbapenemase may be responsible for the carbapenem resistant phenotypes found in *A. baumannii*. However, the findings of resistant genes from two settings (the central and northern part of Thailand) were

different whereas the isolates from the other regions have not been evaluated. Furthermore, the other important genes e.g. VIM, SIM or other OXA-subtypes also have not been studied in *A. bauamannii*. Thus, genes which responsible for CR-*A. baumannii* in Thailand must be identified.

In our institution, Songklanagarind Hospital, a university-affiliated medical school, the problems of antibiotic resistance in gram-negative bacilli has been increasing. Particularly, CR-*A. baumannii* strains, the mortality rate of imipenem-resistant *A. baumannii* (IR-*A. baumannii*) infection in our institution is a high important problem. Jamulitrat et al. investigated patient mortality outcome between July 2003 and September 2005. This cohort study revealed that the mortality rate in the patients with IR-*A. baumannii* compared to imipenem-sensitive *A. baumannii* was 33.8% and 24.1% respectively (Jamulitrat, Thongpiyapoom, & Suwalak, 2007). For the sensitivity of antibiotics, during the five-year period, the percentage of imipenem-sensitive *A. baumannii* strians have been declined from 97 percents in 2001 to 40 percents in 2007 whereas the sensitivity to cefoperazone/ sulbactam remained over 90 %. On the contrary, the other university-affiliated medical schools reported the tendency of percent resistance to imipenem and cefoperazone/ sulbactam in the same way. For instance, the percent susceptibility of imipenem and cefoperazone/ sulbactam in 2006 at Phramongkutklao hospital, Ramathibodi hospital, Rajavithi hospital were approximated 23 and 39, 38 and 58, and 25 and 53, respectively ("MIMS antimicrobial guide," 2007). Like the report of drug susceptibility from national antimicrobial resistance surveillance center in 2006, the susceptible *A. baumannii* to imipenem and cefoperazone/ sulbactam also were 49% and 58%, respectively. Because of the different pattern of drug resistance, the strains, which were isolated from patients from our institution, might differ from the other hospitals and might result in the different choices of effective regimens.

At Songklanagarind Hospital, there are no data to identified resistant genes or the diverse mechanism of antimicrobial resistance in CR-*A. baumannii* and thus selection of the best combination of antibiotics to treat these infections is problematic. Thus, our study aims to identify both resistant genes in OXA and IMP group. The findings of this study will explain the mechanism of resistance and may give the useful data for treatment of *CR-A. baumannii* infections.

1.2. Objectives

The objective of this study is to identify types of IMP and OXA resistant genes in carbapenem-resistant *Acinetobacter baumannii* clinical isolates at Songklanagarind Hospital by polymerase chain reaction technique.

1.3. Scope of Research

In this study, at least 35 clinical isolates from Songklanagarind hospital were used for identification of 8 carbapenemase genes including IMP1, OXA-23, OXA-24, OXA-40, OXA-58, SIM-1, VIM-1 and VIM2 using polymerase chain reaction techniques and DNA sequencing machine.

1.4. Literature Reviews

1.4.1. Microbiology of *Acinetobacter baumannii*

Acinetobacter baumannii, which was classified as members of the genus *Acinetobacter*, is gram-negative coccobacilli, oxidase-negative, with guanine and cytosine base content of 39 to 47 mol%, and non-motile. Usually, *Acinetobacter* have a rod cell size about 1.0 to 1.5 by 1.5 to 2.5 μm in the logarithmic phase of growth but become cocci shape in the stationary phase, arranging in pair or cluster. During 1980s, Bergey's Manual of Systematic Bacteriology classified the genus *Acinetobacter* in the family *Neisseriaceae* as *Acinetobacter calcoaceticus*. This specie has been subdivided into two subspecies, subsp. *anitratus* and subsp. *iwoffii*, but this systemic arrangement has not been widely used. Currently, the development of genetics has resulted in arrangement of new family as *Moraxellaceae*, which has three genus including *Moraxella*, *Acinetobacter*, *Psychrobacter* (Bergogne-Berezin & Towner, 1996). Seven genomic species have already been identified and named i.e. *A. calcoaceticus* (group1), *A. baumannii* (group 2), *A. haemolyticus* (group4), *A. junii* (group 5), *A. johnson* (group 7), *A. iwofii* (group 8), *A. radioresistens* (group 12), with, twelve of the genomic species yet to be named. Mostly, genomic species, *A. baumannii* (group 2), *A. calcoaceticus* (group1), group 3, and group 13TU are isolated from clinical sources (Bergogne-Berezin & Towner, 1996; Lim, et al., 2007).

The virulence of *Acinetobacter* has been hypothesized in four characteristics including (i) the presence of a polysaccharide capsule expresses the surface of strains more hydrophilic property; (ii) the capability of adhesion to human epithelial cells with fimbriae and/or capsular polysaccharide; (iii) the production of enzymes which may damage tissue lipids; and (iv) the potentially toxic role of the lipopolysaccharide component and lipid A on its cell wall (Bergogne-Berezin & Towner, 1996).

For the persistence in the environment**,** various studies have investigated the presence of *Acinetobacter* spp. in the hospital environment, but rates of positive cultures may be different. Rosenthal et al. conducted the study of sources of *Acinetobacter* species found in human culture materials. The results of study revealed that *Acinetobacter* spp. has been found in 27% of hospital sink traps and 20% of hospital floor swab cultures (Rosenthal, 1974). Moreover, Crombach et al. investigated the hospital environment that be collected from an ICU, 12 (11.5%) of 104 air samples from wards harboring colonized patients with *Acinetobacter* spp. and 13 (17%) of 75 samples from bedside cupboards in the same areas (Crombach, Dijkshoorn, van Noort-Klaassen, Niessen, & van Knippenberg-Gordebeke, 1989). Persistence in environment was identified for up to 13 days after the discharge of the patients. Moreover, The duration of *Acinetobacter* spp. outbreak disseminated in patients who admitted to a burn unit was over a 21-month period (Bergogne-Berezin & Towner, 1996). Thus, *Acinetobacter* spp. has dominated characteristics among nosocomial bacteria that favor their persistence in the hospital environment.

A. baumannii plays an important role in nosocomial infections e.g. ventilator associated pneumonia, bacteremia, urinary tract infection, meningitis, peritonitis, and rare cases of endocarditis. Occasionally, *Acinetobacter spp*. has been obtained from the oral cavity, respiratory tract, or skin of healthy subject which is colonization (Bergogne-Berezin & Towner, 1996). Today, the problem of resistant *A. baumannii* is increasing especially, multi-drug resistant strains. Several studies have documented the mechanism of resistace of *A. baumannii* via enzymatic lysis, losing of porins, efflux pump, or changing of receptor of drug. This high level of resistance of *A. baumannii* presents the clinical challenges of appropriate treatment and infection control to reduce infection rates.

1.4.2. Mechanisms of drugs resistance in *A. baumannii*

Regarding to the problems of MDR-*A. baumannii*, several studies have documented the multi-mechanism of resistance of *A. baumannii*. Resistance occurs via enzymatic lysis (including OXA, metalo-β-lactamase, Amp-C, Aminoglycoside-modifying enzyme), losing of porins, efflux pumps, or changing of receptor of drug (penicillin-binding proteins (PBPs), ribosomal RNA, or DNA gyrase). For the mechanism of resistance to β-lactam antibiotics, *A. baumannii* uses all four strategies including enzymatic lysis, losing of porins, efflux pumps, and alteration of target to oppose the antimicrobial activity (Poirel & Nordmann, 2006).

The carbapenemase which has activity in cleavage of the β-lactam ring of carbapenems, cephalosporins and penicillins, have been categorized into two major subtypes due to molecular structure and the essential co-factors to activate the reaction including OXA-carbapenemases and MBL.

According to Bush-Jacoby Medeiros system, OXA-type carbapenemase have been classified by molecular structure in group D β-lactamases or by functional or substrate group in class 2d. Currently, 121 variants of class D β-lactamases have been described on the evidence of the sequence amino acids of enzyme and forty-five of these have carbapenem-hydrolyzing property, which have been named OXA-type carbapenemases. There are eight subgroups in OXA-type carbapenemases and only four of the eight clusters have been verified in *A. baumannii*. The first subgroup includes OXA-23, OXA-27, and OXA-49 (Figure 1). The second subgroup consist of 4 enzymes including OXA-24, -25, -26, and -40. The third group has the OXA-51 family which are intrinsically found in *A. baumannii* whereas only OXA-58 members are in the fourth group (Poirel & Nordmann, 2006; Walther-Rasmussen & Hoiby, 2006).

Valenzuela et al. conducted a study of gene transfer in outbreak of CR-*A. baumannii* in the intensive care unit of Westmead Hospital (Sydney, Australia) between 1995 and 2000. CR-strains (n=13) which were verified, had the *bla*OXA-23 gene and the *bla*OXA-51-like genes (Valenzuela, et al., 2007).

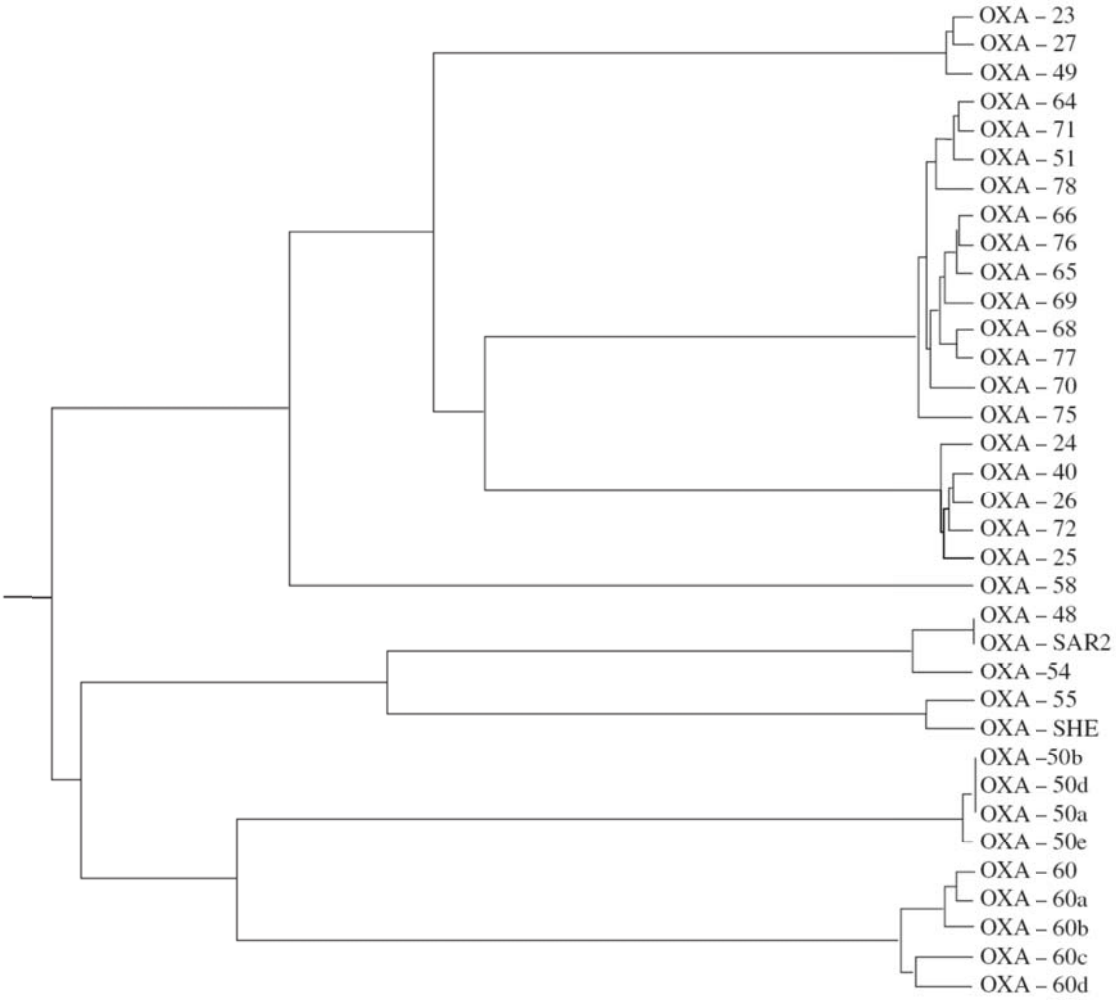

Figure 1. Dendrogram of precursor OXA-type carbapenemases (modified from (Walther-Rasmussen & Hoiby, 2006)

Zarrilli et al. investigated the molecular epidemiology of multidrug-resistant *A. baumannii* that occurred between June 2003 and June 2004 in a tertiary-care hospital in Naples, Italy. Genotypic analysis of 45 available *A. baumannii* isolates was divided into two distinct pulsed-field gel electrophoresis (PFGE) patterns. A blaOXA-51-like gene was amplified from all clinical isolates of PFGE types 1 and 2, while a blaOXA-58-like gene was only identified from isolates of PFGE types 1 and 1a, but not from PFGE type 2. With PCR analysis of MBL genes (including; IMP, VIM, and SIM-type), blaOXA- 23-type and blaOXA-24-type have not been identified (Zarrilli, et al., 2007). This finding is similar to Poirel 's study emphasized the importance of the carbapenemase gene among *A. baumannii* isolates in

Greece between November 2003 and May 2005. Twelve isolates producing the OXA-58 were recovered from patients in the pediatrics hospital (Poirel & Nordmann, 2006).

In the United States, the CR-*A. baumannii* related to OXA-40 carbapenemase. Forty two IR-*A. baumannii* isolates varied in level of carbapenem resistance (imipenem MICs, 64 to 128 µg/ml). PCR was performed with primers for the *bla*OXA-23-, *bla*OXA-40-, and *bla*OXA-58 oxacillinases. Only an OXA-40 gene was amplified in all of carbapenem resistance isolates (Lolans, et al., 2006).

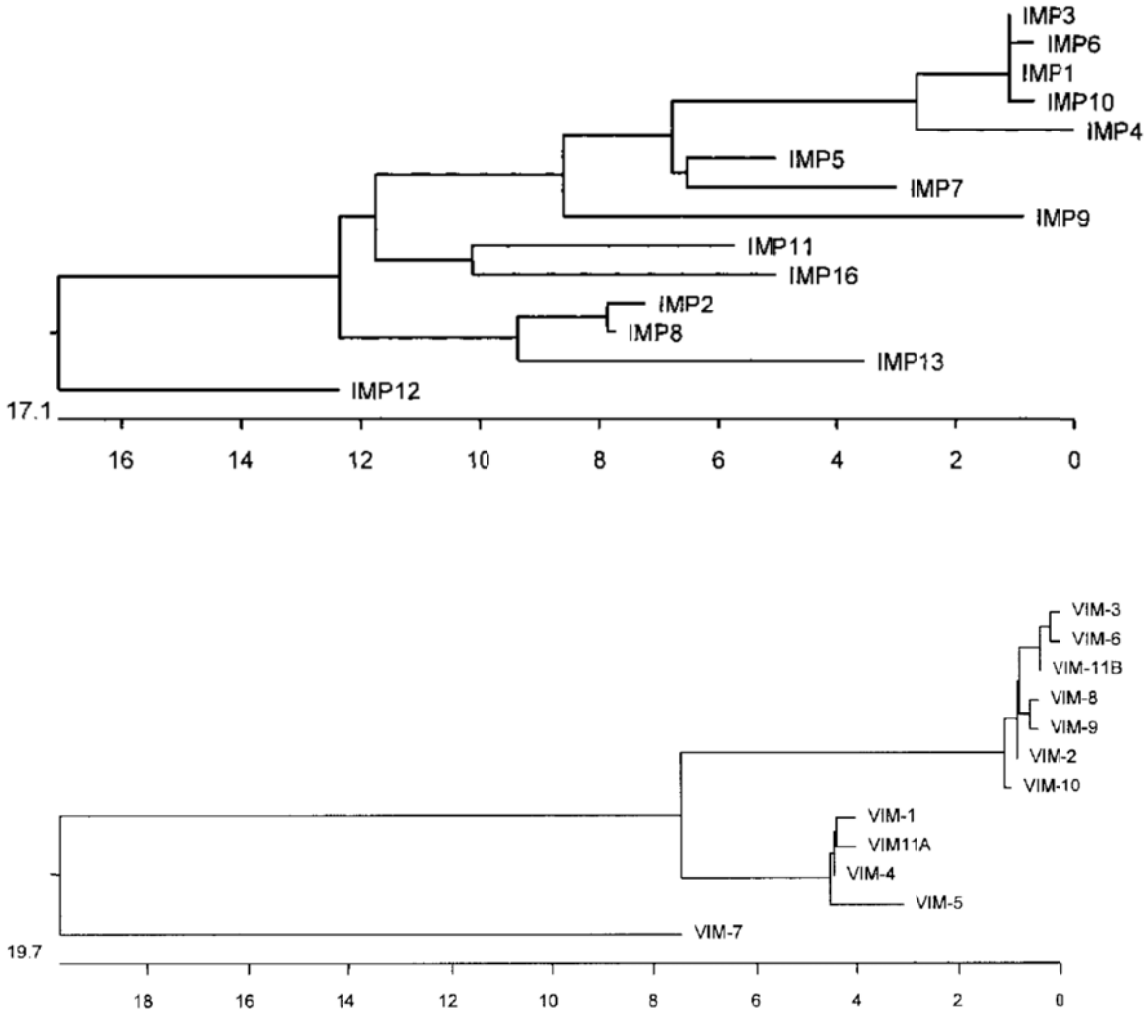

Figure 2. Phylogenies of IMP-and VIM-type MBLs (modified from (Walsh, et al., 2005)

MBLs, like Oxa-carbapenemase, can hydrolyze carbapenems and virtually all other β-lactams. Although, MBLs need Zinc for their action whereas Oxa-carbapenemase act by a serine based mechanism. Because Zinc is an important cofactor,

MBLs are susceptible in vitro to EDTA inhibition thus providing method of identification. Using E-test strips or disk containing imipenem with or without EDTA indicate the MBL producing-organism. At a molecular level, MBLs are classified by structure in group B or by functional or substrate group in class E of Bush-Jacoby Medeiros system. To date, three MBLs have been identified in *A. baumannii*, IMP-like, VIM-like (Figure 2), and SIM-1 (Poirel & Nordmann, 2006; Walsh, et al., 2005).

The IMP and VIM variants show a high level of carbapenem resistance, because of their strongly hydrolytic property against β-lactam antibiotics (Poirel & Nordmann, 2006). The epidemiologic studies of MBL producing-*A. baumannii* have been investigated. Lim et al. identified 40 isolates of clinical *A. baumannii* isolated from patients admitted at a university hospital between August 2004 and March 2005. Only a single isolate (2.5%) was MBL producers carrying IMP-1 by PCRs whereas, VIM-type MBLs were negative (Lim, et al., 2007). Contradictory, the study of Wroblewska et al. found that PCRs for IMP-type MBLs were negative, but VIM-type MBLs were positive in carbapenem-resistant isolates belonging to patients in Poland during 2003–2005 (Wroblewska, et al., 2006).

2. Methodology

2.1. Culture media

- Broth agar with glycerol
- Macconkey agar (Difco)
- Blood agar base (Difco)
- Mueller-Hinton broth (Difco)
- LH broth and agar

2.2. Chemicals and Kits

- Sterile distilled water
- Sterile 0.9% Sodium chloride
- Illustra plasmidPrep Mini Spin Kit (GE health science)
- DNeasy mini kit (Qiagen)
- deoxy-ribonucleotide triphosphate (dNTP
- $MgCl_2$ in 5X GoTaq® buffer
- GoTaq® polymerase
- PCR purification kit (Promega, USA)
- pDrive vector (Qiagen)
- *E. coli* TOP10
- *Restriction enzyme Dra* I, *Eco R*I, *Pst* I

2.3. Instruments and Equipments

- Inoculation loop
- Disposable Petri dish # 94 X 16 mm
- Test tube # 130 X 100 mm. with plastic cap
- Autoclavable plastic tube # 50 mL with plastic cap
- Sterile 96-well plate type U shape

- Erlenmeyer flask with cap # 250 and 500 mL
- Measuring pipettes # 5, 10, 25 mL
- Takara thermocycler gradient (TaKaRa, Japan)
- agarose gel electrophoresis
- UV transillumination (312 nm)
- Automatic DNA sequencer

2.4. Clinical isolates

The clinical isolates are obtained from patients with CR-*A. baumannii* infections. The first isolates which obtained from patients, are tested; the other following isolates in the same patient are excluded.

2.5. Sample size

The previous studies have been investigated the proportion of carbapenemase-gene in different values. In Thailan, Boonkerd et al. reported that OXA23 has been found 15 of 16 (93.75%) of clinical imipenem-resistance *A. baumannii*. Besides, Duangseesi et al. have been also detected about 37.5% of them (Boonkerd, et al., 2009; Duangseesai, et al., 2005). Thus, the proportion is used for calculation the sample size, which are 37.5 or 93.75%. The sample size for investigating the carbapenemase-gene may be taken to equal

$$n = \frac{z^2 P(1 - P)}{d^2}$$

By,

n = Sample size (no of stains)

P = Population proportion that has required characteristics (the proportion of interesting gene in carbapenem resistance *A. baumannii*; 0.37 or 0.94)

Z = Acceptable level of confidence in standard error (1.96)

d = Allowed level of sampling error (0.10)

Suggesting that a total sample of 90 (based on prevalence at 0.37) or 217 (based on prevalence at 0.94) imipenem-resistant *A. baumannii* isolates should be studied. However, we cannot collect the sample as above calculation because the total isolate which meet the criteria of the international sepsis forum consensus conference on definitions of infection in the intensive care unit and International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics, were gathered about 30 strains in 2008. Thus, we need to use the period for collecting the organisms about 3-7 years. If we take a long time to gather the isolates, we think that the results of this study may be out of date and cannot apply the data to represent the real situation of resistant. Thus, in this study, we will gather all of isolates that meet criteria of infection and occur during January-December 2008

The clinical isolates are tested by EDTA inhibition testing in order to detect the phenotypic of metalo-β-lactamase (MBLs) producer. Briefly, spread the organism into Muller Hinton agar plate and then put two disk of imipenem on the agar plate. The first disk of imipenem is dropped with EDTA and incubates the agar plate at 35 ^{O}C for 18 hours. The diameter of clear zone of imipenem disk with EDTA must be larger size over 7 mm than the disk, which has only imipenem. This result indicates that the tested strain can produce MBL (Yong et al., 2002).

2.6. Isolation of plasmid and genomic chromosomal DNA

Plasmids of *A. baumanii* clinical isolates are prepared using Illustra plasmidPrep Mini Spin Kit (GE health science) according to manufacturing protocol. The genomic DNAs of *A. baumanii* clinical isolates are prepared using DNeasy mini kit (Qiagen).

2.7. Primer design

The couple primers of 8 drug resistant genes including IMP-1, OXA-23, OXA-24, OXA-40, OXA-58, SIM-1, VIM-1 and VIM-2 are used for amplification covering the whole genes. Each primer set is designed from nucleotide sequence of each gene retrieved from GeneBank. The accession numbers of each gene are shown in Table 1

Table 1. The oligonucleotide primers designing from resistance genes data in *A. baumannii*.

Gene	Accession number	Primer information F = forward R = reverse	Size of PCR product (bp)
IMP-1 IMP-2 IMP-4 IMP-5 IMP-8 IMP-11 IMP-19	AJ640197 AJ243491 AY590475 AF290912 EF127959 AB074436 AB184977	F – 5`GGAATTCCATGARSAARTTATYTGTWTT3` R – 5`CGGGATCCTTAGTTRCTTRGYTKTGATGG3	741
OXA-23	AJ132105	F – 5`GGAATTCCATGAATAAATATTTTA3` R - 5`GGATCCCGTTAAATAATATTCAGC3`	822
OXA-24	AJ239129	F – 5`GGAATTCCGTACTAATCAAAGTTGTGAA3` R - 5`GGATCCCGTTCCCCTAACATGAATTTGT3`	828
OXA-40	AF509241	F – 5`GGAATTCCATGAAAAAATTTATAC3` R - 5`GGATCCCGTTAAATGATTCCAAGA3	828
OXA-58	EU107365	F – 5`GGAATTCCATGAAATTATTAAAAA3` R - 5`GGATCCCGTTATAAATAATGAAAA3`	843
SIM-1	AY887066	F – 5`GGAATTCCATGAGAACTTTATTGA3` R - 5`GGATCCCGTTAATTAATGAGCGGC3`	741
VIM-1	DQ112355	F – 5`GGAATTCCATGTTAAAAGTTATTA3` R - 5`GGATCCCGCTACTCGGCGACTGAG3`	801
VIM-2	AF291420	F – 5`GGAATTCCATGTTCAAACTTTTGA3` R - 5`GGATCCCGCTACTCAACGACTGAG3`	801

2.8. Gene amplification

Each gene is amplified by PCR technique. The PCR mixture consists of 1 µL of either plasmid or genomic DNA as PCR template, 10 mM each of forward and reverse primers, 0.2 mM each deoxy-ribonucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$ in 5X GoTaq® buffer. The volume of GoTaq® polymerase is 1.25 units. The total volume of PCR mixture is 50 µL. The thermocycling is carried out on Takara thermocycler gradient (TaKaRa, Japan), Starting with denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute then 1 minute of annealing temperatures, which should be optimized for each primer set, based on melting temperature (Tm) of the primer and 72°C for 2 minutes, with final extension at 72°C for 10 minutes

The PCR products are then applied to agarose gel electrophoresis, stained with SYBR-safe DNA straining (Invitrogen). The bands of nucleotides were detected by UV transillumination (312 nm).

2.9. Plasmid construction and amplification

The PCR products are purified from low melting point agarose gel electrophoresis and PCR purification kit (Promega, USA) following the product instructions. The purified PCR products are ligated into pDrive vector (Qiagen) and amplified by transformation into *E. coli* TOP10. After overnight culture, a single colony is inoculated in to LB-liquid media supplement with ampicillin. The plasmid from overnight *E. coli* culture is then purified and used for sequencing analysis.

2.10. PCR product analysis by restriction enzymes

Several restriction enzymes such as *Dra* I, *Eco R*I, *Pst* I are used for digestion of PCR product into specific length fragments. Different fragment patterns indicate some mutation of genes which can be used as a shortcut for identified new genes.

2.11. DNA sequencing analysis

The amplified DNA or recombinant plasmid will be sequenced by automatic DNA sequencer (Central scientific measurement center, Prince of Songkla University, Songkhla, Thailand). The sequenced nucleotides will be interpreted using “Bioedit” software and compared with the known genes reported in the Genbank database at the National Center for Biotechnology Information (NCBI) website.

3. Result and Discussion

3.1. Identification of resistant genes and in CRAB

3.1.1. Characterization and antimicrobial susceptibility test of CRAB strains

Among the 110 CRAB isolates at Songklanagarind hospital during the study the period, twenty two strains (20%) were obtained from sterile sites including blood (n=14), intrabdominal tissues (n=3), CSF (n=2), ascites fluids (n=2) and other sites (n=1) (Table 2). All isolates were resistant to ampicillin, piperacillin/tazobactam, ceftazidime, cefipime, imipenem, meropenem and ciprofloxacin. The percentage of susceptible strains varied for trimethoprim/sulfamethoxazole (9.1%), gentamicin (22.7%), amikacin (27.3%), cefoperazone/sulbactam (86.4%) and colistin (100%). According to EDTA inhibition testing, no one indicates that the tested strains could produce MBL.

3.1.2. Detection of OXA and MBL carbapenemase genes in CRAB

The twenty-two isolates from sterile site were amplified for determination of resistant gene. All isolates carried the $bla_{OXA\text{-}23\text{-likes}}$ (Fig. 3) but did not carry the OXA and MBL genes ($bla_{OXA\text{-}40}$, $bla_{OXA\text{-}58}$, $bla_{IMP\text{-}like}$, $bla_{SIM\text{-}1}$, $bla_{VIM\text{-}1}$ and $bla_{VIM\text{-}2}$) (Table. 2 and Fig. 4).

Table 2. Characteristics of the 22 carbapenem-resistant *A. baumannii* (CRAB) isolates and amplification results of each gene

No	Strains	Specimen	Oxa-23	Oxa-24	Oxa-40	Oxa-58	IMP_{like}	SIM-1	VIM-1
1	AB11	Blood	✓	×	×	×	×	×	×
2	AB13	Ascitic fluid	✓	×	×	×	×	×	×
3	AB15	Pancrease tissue	✓	×	×	×	×	×	×
4	AB23	Blood	✓	×	×	×	×	×	×
5	AB54	Intra-Abdominal tissue	✓	×	×	×	×	×	×
6	AB58	Intra-Abdominal tissue	✓	×	×	×	×	×	×
7	AB77	Blood	✓	×	×	×	×	×	×
8	AB79	Blood	✓	×	×	×	×	×	×
9	AB133	Blood	✓	×	×	×	×	×	×
10	AB153	Blood	✓	×	×	×	×	×	×
11	AB164	Blood	✓	×	×	×	×	×	×
12	AB167	Blood	✓	×	×	×	×	×	×
13	AB179	Blood	✓	×	×	×	×	×	×
14	AB198	Cerebrospinal fluid	✓	×	×	×	×	×	×
15	AB271	Other	✓	×	×	×	×	×	×
16	AB272	Blood	✓	×	×	×	×	×	×
17	AB286	Blood	✓	×	×	×	×	×	×
18	AB307	Blood	✓	×	×	×	×	×	×
19	AB313	Ascitic fluid	✓	×	×	×	×	×	×
20	AB315	Blood	✓	×	×	×	×	×	×
21	AB316	Blood	✓	×	×	×	×	×	×
22	AB322	Cerebrospinal fluid	✓	×	×	×	×	×	×

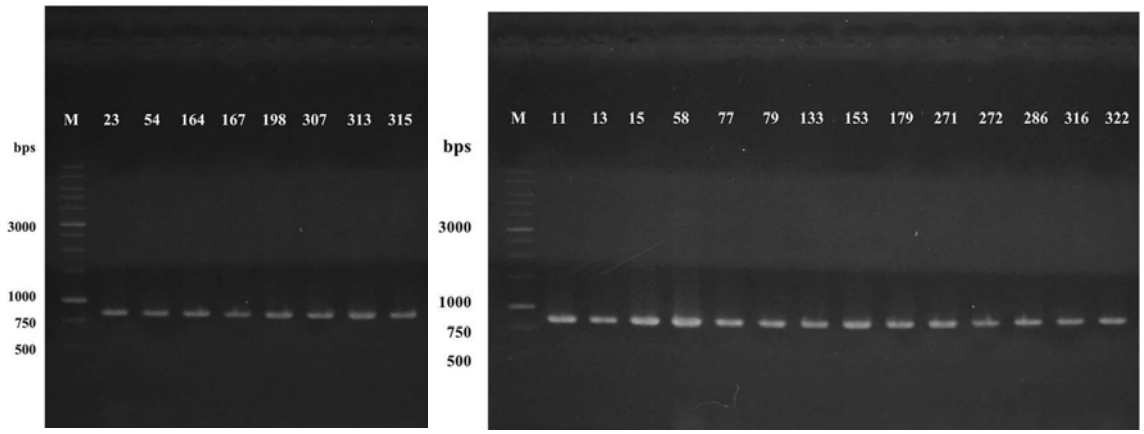

Figure 3. The presence of 822-base pair-OXA-23 gene in plasmid DNA content of twenty-two CRAB isolates. M, DNA marker (SibEnzyme); the numbers above each lane indicate the CRAB strain. The number on the left panel indicated the molecular weight marker in base pairs (bps) unit.

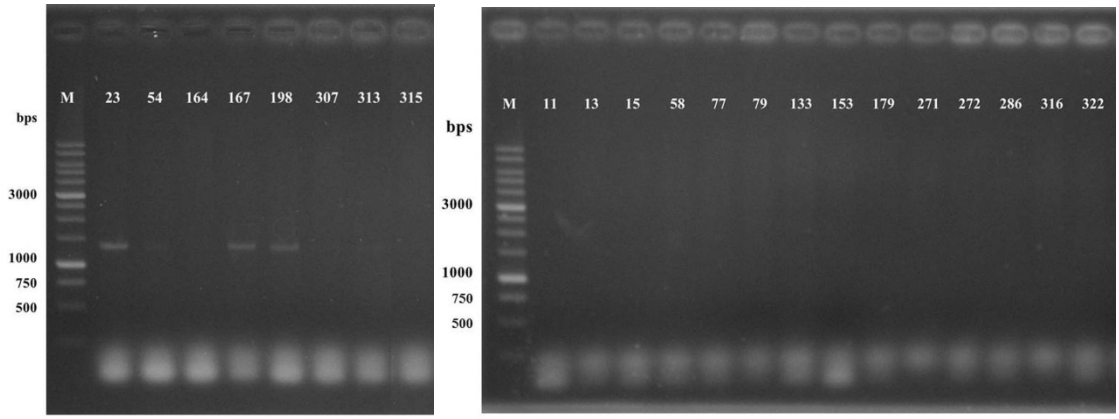

Figure 4. The absent of 828-base pair-OXA-24 gene in genomic DNA content of twenty-two CRAB isolates. M, DNA marker (SibEnzyme); the numbers above each lane indicate the CRAB strain. The number on the left panel indicated the molecular weight marker in base pairs (bps) unit.

Among 22 strains producing OXA-23 likes carbapenemase, the sequencing analysis indicated that sixteen isolate carried *bla*$_{OXA-23}$ genes and 6 isolates harboring new variant *bla*$_{OXA-23-likes}$ genes named as OXA-165, OXA-166, OXA-167, OXA-169, OXA-170 and OXA-171 (GenBank accession number HM488986, HM488987, HM488988, HM488990, HM488991 and

HM488992), respectively. The sequence identities among novel enzyme of each gene greater than 99% homologous to *bla*$_{OXA-23}$. The position of a variant amino acid was shown in Table 3. The IS*Aba1* element was found as upstream of the *bla*$_{OXA-23}$ and *bla*$_{OXA-23-likes}$ genes.

Table 3 Amino-acid changes yielding 6 novel enzymes related to OXA-23 carbapenemase

The changed amino-acid [a]	The novel enzyme
Isoleucine $_{223}$ ⟶ Valine	OXA-165
Valine $_{52}$ ⟶ Alanine	OXA-166
Leucine $_{263}$ ⟶ Methionine	OXA-167
Lysine $_{47}$ ⟶ Arginine	OXA-169
Glutamate $_{207}$ ⟶ Lysine	OXA-170
Isoleucine $_{258}$ ⟶ Threonine	OXA-171

[a] The amino acid sequences among OXA-165, -166, -167, -169, -170 and -171 were compared with those of OXA-23 showing >99% identity. Position of changed amino acid defined by number based on OXA-23 amino-acid sequence.

4. Discussion and conclusion

Nosocomial infections due to CRAB are increasing worldwide (Hsueh, et al., 2005; Marshall et al., 2007). Treatment of CRAB infection is challenging due to multi-mechanisms of drug resistance. *A. baumannii* possesses various mechanisms of antibiotic resistance including via enzymatic lysis (including OXA, metallo-β-lactamase, Amp-C, aminoglycoside-modifying enzyme), loss of porins, efflux pumps, or via a changein drug receptor (penicillin-binding proteins (PBPs), ribosomal RNA, or DNA gyrase) (Bergogne-Berezin & Towner, 1996; Bonomo & Szabo, 2006). For β-lactam antibiotics, all these resistance mechanisms have been described in *A. baumannii*. Especially problematic is carbapenemase, which causes lysis of the β-lactam ring of carbapenems, cephalosporins and penicillins. Currently, there are four of the eight OXA type carbapenemases clusters have been found in *A. baumannii* in various region of the world including OXA-23, OXA-40, OXA-58, and OXA-51 (intrinsically found in *A. baumannii*) (Walther-Rasmussen & Hoiby, 2006). The prominent OXA-type in this present study finding, OXA-23 has been previously reported by two investigators in Phitsanulok province (upper middle part of Thailand) (Niumsup, Boonkerd, Tansawai, & Tiloklurs, 2009) and Bangkok (Thapa, Tribuddharat, Srifuengfung, & Dhiraputra, 2010). This report adds to these findings by also showing this resistance mechanism in the southern region of Thailand. In contrast to OXA-type, MBLs, IMP-1 carbapenemase, was reported by Niumsup *et al.* in 2 of 13 strains of CRAB (Niumsup, et al., 2009) whereas this present study and Thapa *et al*. did not show CRAB isolates producing IMP carbapenemase (Thapa, et al., 2010). Thus, OXA-23 might play important role as mechanism for resistant to carbapenem in Thailand.

While carbapenemases are the predominate mechanism for carbapenem resistence, the efflux pump and loss of porins has also been shown to occur. Magnet et al. isolated MDR-*A. baumannii* strain BM4454 from a patient with urinary tract infection. This strain encoded the *adeB* gene in RND system (Magnet, Courvalin, & Lambert, 2001). Moreover, Damier-Piolle and Lin *et al*. also reported the discovery of the AdeIJK and

AdeDE, respectively in a clinical isolate (Damier-Piolle, Magnet, Bremont, Lambert, & Courvalin, 2008; Lin, Ling, & Li, 2009). In addition, other efflux pump systems (MATE, and MFS family) that have also been found in *A. baumannii* (Vila, Marti, & Sanchez-Cespedes, 2007). However, our finding in this present study was not to study the efflux pump expression in CRAB from Thailand.

As previously reported, carbapenem resistant bactertial isolates had reduced expression of porins with sizes 22, 33-36, 37-, 44-, and 47-kDa in OMP analysis compared with carbapenem-susceptible isolates (Bou, Cervero, Dominguez, Quereda, & Martinez-Beltran, 2000; Clark, 1996; Limansky, Mussi, & Viale, 2002; Quale, Bratu, Landman, & Heddurshetti, 2003). However, our study limited to study the presence of OXA-23 carbapenemase, putative OMP may be one of resistant mechanisms in CRAB isolates that were found in the previous studies.

There were certain limitations of this present study including 1) we identified only the most common mechanisms in CRAB and did not study changing of PBP, porins losing and efflux pump as one of mechanism of resistance in CRAB isolates (Fernandez-Cuenca et al., 2003; Gehrlein, Leying, Cullmann, Wendt, & Opferkuch, 1991). 2) We amplified the gene encoding carbapenemase in twenty two strains of true pathogen; the low prevalence of resistant genes might not be detected. However, according to Thapa and colleague study revealed the absence of MBL or other OXA carbapenemases in 200 strains of CRAB (Thapa, et al., 2010). Further study is required for confirmation of this hypothesis. 3) With the presence of new six variant OXA, the position of single amino acid was changed from OXA-23 in each enzyme. This mutation did not possibly affect activity due to outside of the S-T-F-K_{69-72}, F-G-$N_{152-154}$ and K-T-$G_{216-218}$ showing active site regions of enzyme (Brown & Amyes, 2006; Donald, Scaife, Amyes, & Young, 2000). However, these enzymes might be further characterized the capacity and the kinetic measurements of cleavage of β-lactam antimicrobial agents.

In conclusion, this is the report of the important resistant mechanism of carbapenem via producing of OXA-23 carbapenemase in CRAB isolate from Thailand. We identified six novel of OXA-23like enzyme that needs to be further studied.

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6. ผลงานตีพิมพ์ที่เกี่ยวข้องกับงานวิจัยนี้

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Manuscripts

Santimaleeworagun, W., Wongpoowarak, P., Chayakul, P., Pattharachayakul, S., Tansakul, P. and Garey, K.W. (*2013*) Loss of the 29-kilodalton-Porin and OXA-23-like Carbapenemase in Clinical Isolates of Carbapenem Resistant *Acinetobacter baumannii* from Songklanagarind Hospital, Thailand: Preliminary Report of Six Novel OXA-23-like enzymes (In preparation)