

IDENTIFICATION AND CHARACTERIZATION OF CARBAPENEMASE GENES IN CLINICAL ISOLATES OF CARBAPENEM-RESISTANT *ACINETOBACTER BAUMANNII* FROM A GENERAL HOSPITAL IN THAILAND

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Abstract. This study identified, using PCR *bla*_{OXA-23} encoding carbapenemase OXA-23 in 42 carbapenem-resistant *Acinetobacter baumannii* (CRAB) clinical isolates and *bla*_{OXA-40-like} encoding carbapenemase OXA-40-like in one isolate (the first such Thai sample) obtained from patients admitted to Hua Hin Hospital, Thailand during November 2011-September 2012. Using repetitive extragenic palindromic PCR, the isolates could be divided into 4 different clones, with 40 isolates belonging to clone A and the remaining to B, C and D. Other resistance in CRAB needs to be investigated.

Keywords: *Acinetobacter baumannii*, carbapenem resistance, carbapenemase gene

INTRODUCTION

Nosocomial infection with *Acinetobacter baumannii* is one of the global major problems due to its antimicrobial drug resistance and its ability to cause infections in various organs (Kempf and Rolain, 2012; Zarrilli *et al*, 2013). In the United States, a surveillance study of 24,179 patients with bloodstream infection found that *A. baumannii* results in the second most common cause of mortality (43.4%) in patients admitted to intensive care units (Wisplinghoff *et al*, 2004). Similarly, in

Spain *A. baumannii* is the third cause of infection in mechanically ventilated patients (Alvarez-Lerma *et al*, 2007). Likewise in Taiwan, as in other regions of the world, the rate of infection due to *A. baumannii* in hospitals has also increased from 25 per 10,000 patients in 1999 to 55 per 10,000 patients in 2003 (Hsueh *et al*, 2005). In Thailand, according to the national antimicrobial resistance surveillance report for the year 2009, *A. baumannii* isolated from sputum and other specimens ranked as the third and fourth most common pathogen, respectively (NARST, 2007).

Besides being a major cause of infection, multidrug resistant *A. baumannii* (MDR-AB) has increased significantly the problem of clinical treatment. Dejsirilert *et al* (2009) found that MDR-AB from 28 hospitals participated in the NARST pro-

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gram resistant to amikacin, ciprofloxacin and ceftazidime has increased from 46% in 2000 to 56% in 2005. Moreover, the surveillance program in 33 hospitals throughout Thailand, *A. baumannii* resistant to carbapenems (CRAB) causes significant infection with increasing rate, from 18% in 2000 to 65% in 2005 (Apisarnthanarak *et al*, 2009). MDR-AB and CRAB use multiple mechanisms for resistance to antimicrobial agents, such as producing drug-destroying enzymes (especially against carbapenemases), reducing amounts of drugs into the cells (via porin loss), transporting drugs out of the cell (via efflux pump) and mutating target sites of antimicrobials (Bergogne-Berezin and Towner, 1996; Bonomo and Szabo, 2006).

CRAB containing carbapenemase also destroys penicillin and cephalosporin (Bergogne-Berezin and Towner, 1996; Bonomo and Szabo, 2006). Currently, CRAB is mostly resistant to OXA-type carbapenemases, such as OXA-23, OXA-40, and OXA-58 (Poirel and Nordmann, 2006; Walther-Rasmussen and Hoiby, 2006). However, metallo- β lactamases including IMP, VIM, and SIM are found also in *Acinetobacter* spp (Poirel and Nordmann, 2006).

According to nosocomial CRAB infection, the clonal relationship is crucial to control and prevent the spread of this pathogen. A repetitive extragenic palindromic PCR (REP-PCR) is widely used for molecular typing of *A. baumannii* isolates to amplify putative REP-like elements in the genomic DNA (Bou *et al*, 2000a).

In Thailand, there have been a limited number of studies of carbapenemase encoded by *bla*_{OXA-23} (Niumsup *et al*, 2009; Thapa *et al*, 2010; Santimaleeworagun *et al*, 2011) and of IMP (Niumsup *et al*, 2009).

However, such studies were conducted in large medical schools and the data of resistance mechanism might differ from those in general hospitals due to the patterns of antibiotic use, various drug types and clinical status of patients. Thus, the present study aimed to identify presence of carbapenemases in CRAB isolated from patients admitted to general hospitals using PCR technique to identify their cognate genes and to evaluate their clonal relationship by REP-PCR.

MATERIALS AND METHODS

Bacterial strains

All clinical isolates were obtained from in-patients admitted at Hua Hin Hospital, a 400-bed general hospital in western Thailand during November 2011 to September 2012. Clinical isolates of CRAB were collected from various clinical specimens. CRAB is defined as an isolate resistant to imipenem (10 μ g) and meropenem (10 μ g) according to the disk diffusion assay based on the Clinical and Laboratory Standards Institute (CLSI, 2012). All CRAB isolates were kept at -20°C until tested.

The protocol was approved by the institutional review board of Hua Hin Hospital.

Amplification of carbapenem-resistant genes

Primers for PCR amplification of carbapenemase genes, *bla*_{IMP-like}, *bla*_{OXA-23}, *bla*_{OXA-40}, *bla*_{OXA-58}, *bla*_{SIM-1}, *bla*_{VIM-1} and *bla*_{VIM-2} are listed in Table 1. Chromosomal DNA of clinical isolates was extracted using a commercial kit (RBC Bioscience, San Diego, CA). PCR of 50 μ l consisted of 1 μ l of genomic DNA, 10 mM each primer pair, 0.2 mM dNTPs, 1.5 mM MgCl₂, 5 μ l of 10X PCR buffer and 1.25 U *Taq* DNA

Table 1

Primers, amplicon sizes and annealing temperature used in PCR-based detection of *A. baumannii* carbapenem resistance genes.

Gene	Primer sequence	Size of amplicon (bp)	Annealing temperature (°C)
<i>bla</i> _{IMP-like}	F- 5' CTRCCGCAGSAGMGBCTTTG3'	587	52
	R- 5' AACCCAGTTTTGCHTTACCAT3'		
<i>bla</i> _{OXA-23}	F- 5' GGAATTCATGAATAAATATTTTA3'	822	42
	R- 5' GGATCCCGTTAAATAATATTCAGC3'		
<i>bla</i> _{OXA-40}	F- 5' GGAATTCATGAAAAAATTTATAC3'	828	45
	R- 5' GGATCCCGTTAAATGATTCCAAGA3'		
<i>bla</i> _{OXA-58}	F- 5' GGAATTCATGAAATTATTAATAA3'	843	45
	R- 5' GGATCCCGTTATAAATAATGAAAA3'		
<i>bla</i> _{SIM-1}	F- 5' GGAATTCATGAGAACTTTATTGA3'	741	47
	R- 5' GGATCCCGTTAATTAATGAGCGGC3'		
<i>bla</i> _{VIM-1}	F- 5' GGAATTCATGTTAAAAGTTATTA3'	801	52
	R- 5' GGATCCCGCTACTCGGCGACTGAG3'		
<i>bla</i> _{VIM-2}	F- 5' GGAATTCATGTTCAAACCTTTTGA3'	801	52
	R- 5' GGATCCCGCTACTCAACGACTGAG3'		

polymerase (Invitrogen, Carlsbad, CA). Thermocycler was performed as follows: 94°C for 5 minutes; 30 cycles of 94°C for 45 seconds, annealing temperature specific for each primer pair (Table 1) for 45 seconds, and 72°C for 1 minute; with a final heating at 72°C for 10 minutes. PCR amplicons were separated by 1.0% agarose gel-electrophoresis, stained with ethidium bromide and visualized under UV light. The amplicon sizes were compared with positive controls (*bla*_{OXA-23}, *bla*_{OXA-40}, *bla*_{OXA-58} and *bla*_{SIM-1} in *A. baumannii* producing strains and *bla*_{IMP}, *bla*_{VIM} in *P. aeruginosa* producing strains) and their identities confirmed by DNA sequencing (Ward Medic, Bangkok, Thailand).

REP-PCR

The REP region was PCR amplified in 50 µl mixture consisting of 1 µl of genomic DNA, 10 mM each REP-forward (5'-IIIGC-GCCGICATCAGGC-3') and REP-reverse

(5'-CGTCTTATCAGGCCTAC-3') primers (Bou *et al*, 2000b), 0.2 mM dNTPs, 1.5 mM MgCl₂, 5 µl 10X PCR buffer, and 1.25 U *Taq* polymerase (Invitrogen, Carlsbad, CA). Thermocycling conditions were as follows: 94°C for 10 minutes; 30 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 2 minutes; and a final step at 72°C for 16 minutes. REP-PCR amplicons were separated by 1.2% agarose gel-electrophoresis, stained with ethidium bromide and recorded by a UV transilluminator fitted with a camera. An isolate is classified as a different clone if its REP-PCR pattern differs from the others by ≥ three bands (Bou *et al*, 2000a).

RESULTS

Characteristics and antimicrobial susceptibility of CRAB isolates

A total of 43 CRAB isolates from 43 hospitalized patients were from spu-

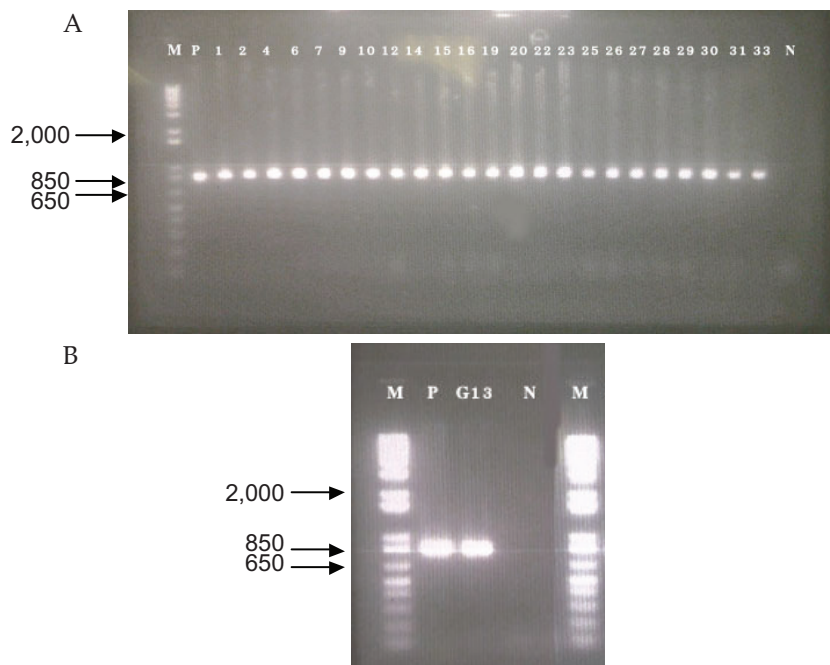


Fig 1—PCR amplification of *A. baumannii* *bla*_{OXA-23} (A) and *bla*_{OXA-40-like} (B) from clinical specimens. Primers and PCR conditions are described in Materials and Methods and Table 1. Lane M: DNA size markers; lane P: positive control; lanes 1, 2, 4, 6, 7, 9, 10, 12, 14, 15, 16, 19, 20, 22, 23, 25, 26, 27, 28, 29, 30, 31, 33 (in A) and G13 (in B): CRAB isolates; lane N: negative control. Numbers on the left margin indicate DNA sizes (bp).



Fig 2—REP-PCR pattern of 15 isolates carbapenem resistant *A. baumannii* from clinical specimens. Primers and PCR conditions are described in Materials and Methods. Lane M: DNA size markers; Clone A: isolate numbers 23, 25, 26, 44, 46, 20, 41, 54, 10, 14, 27, 43; Clone B: isolate number 13; Clone C: isolate number 40; Clone D: isolate number 57. Numbers on the left margin indicate DNA sizes (bp).

tum ($n=31$), pus ($n=7$) and urine ($n=3$). Only 4.7% of the samples were isolated from sterile sites (blood, $n=2$). These CRAB isolates were totally resistant to ceftazidime (30 μ g), imipenem (10 μ g) and meropenem (10 μ g) using the disc diffusion assay. The majority of the isolates were resistant to ciprofloxacin (5 μ g), gentamicin (10 μ g), amikacin (30 μ g), and cefoperazone/sulbactam (75/30 μ g) (95%, 93%, 91% and 95%, respectively).

Detection of OXA and MBL genes

Forty-two CRAB isolates carried *bla*_{OXA-23} (see Fig 1A for representative examples) and only one isolate harbored *bla*_{OXA-40-like} (Fig 1B). However, there were no *bla*_{OXA-58}, *bla*_{IMP-like}, *bla*_{SIM}, *bla*_{VIM1} or *bla*_{VIM2} among the CRAB isolates (data not shown). The identity of *bla*_{OXA-23} was confirmed by DNA sequencing and comparing with GenBank database. The *bla*_{OXA-40-like} also was sequenced and found to be 99% similar to *bla*_{OXA-40} (GenBank accession no. KJ748571).

Clonal relationship among CRAB isolates

REP-PCR profiles revealed that the CRAB isolates could be classified into 4 clones, A, B, C and D (Fig 2). Most strains ($n = 40$) belonged to clone A, and the remaining three to clones B, C and D (data not shown).

DISCUSSION

CRAB infections have become a major problem worldwide (Zarrilli *et al*, 2013). In addition, this pathogen has diverse mechanisms of resistance to antibacterial agents (Bonomo and Szabo, 2006). One mechanism in CRAB is the production of carbapenemases capable of degrading all β -lactam antibiotics. Although usually both genes encoding OXA and MBL carbapenemase genes are found in CRAB strains, mostly only *bla*_{OXA} is present (Poirel and Nordmann, 2006; Walther-Rasmussen and Hoiby, 2006). Currently, there exist four of eight types of OXA carbapenemases worldwide, but mainly OXA-23, -40, -51, and -58 (Walther-Rasmussen and Hoiby, 2006).

Our results showed that OXA-23 was predominant in CRAB isolated from in-patients of a general hospital located in western Thailand. OXA-23 is the major type reported in Bangkok (central Thailand), Phitsanulok (northern Thailand) and Songkhla (southern Thailand) (Niumsupet *et al*, 2009; Thapa *et al*, 2010; Santimaleeworagun *et al*, 2011). Similar to this study, MBL-type could not be found among clinical CRAB isolates from Bangkok and Songkhla, Thailand (Thapa *et al*, 2010; Santimaleeworagun *et al*, 2011).

This is the first report of *bla*_{OXA-40-like} in Thailand. The presence of *bla*_{OXA-40} is frequent in European countries, such as Spain (Ruiz *et al*, 2007; Villalon *et al*, 2013)

and Portugal (Quinteira *et al*, 2007), and in the United States (Lolans *et al*, 2006), but is seldom found in Poland (Nowak *et al*, 2012), Egypt (Al-Hassan *et al*, 2013), Iran (Sohrabi *et al*, 2012) and South Korea (Lee *et al*, 2009). The origin of the Thai *bla*_{OXA-40-like} needs further investigation.

As regards the clonal relationships of CRAB isolates, in this study, clone type A accounted for 93% of the isolates, suggesting the ongoing survival of this lineage in the hospital environment. Shertztz and Sullivan (1985) showed that the duration of an *Acinetobacter* spp outbreak dissemination among patients who were admitted to a burn unit is over a period of 21 months. Thus, an efficient infection control program can minimize the reservoir for bacterial transmission in a hospital setting (Choi *et al*, 2010).

Although we have examined only one of the various resistance mechanisms of CRAB, it appears that the major cause of antibacterial resistance is due to the presence of carbapenemase OXA-23. It may be argued that the sample size (43) is small, but Thapa *et al* (2010) reported that 200 clinical CRAB samples had no MBL or other OXA type except OXA-23 carbapenemase. Nevertheless, one CRAB isolate did carry *bla*_{OXA40-like} so surveillance of resistant mechanisms in *A. baumannii* needs to be continually conducted.

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