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 EcoRI-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 $bla_{OXA23} + bla_{VIM2}$ ($n = 20$), $bla_{OXA23} + bla_{NDM1}$ ($n = 2$), $bla_{VIM2} + bla_{VIM}$, and $bla_{NDM1}$ ($n = 1$) 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, $bla_{OXA23}$, $bla_{OXA40}$, $bla_{OXA58}$, $bla_{VIM}$, $bla_{SIM}$, $bla_{NDM}$, 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,
Metallo-β-lactamase are carried via mobile genetic elements (Poirel and Nordmann, 2006; Tsakris et al, 2006; Chen et al, 2011a). For example, bla\textsubscript{VIM2} cassettes are inserted into class 1 integron in CRAB isolated from patients in Greece (Tsakris et al, 2006). More recently, bla\textsubscript{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 interspecies of bacteria (Walsh et al, 2011).

Previous studies in Thailand reported CRAB isolates carrying bla\textsubscript{OXA23} (Mendes et al, 2009; Niumsup et al, 2009; Higgins et al, 2010) and one study revealing an isolate carrying bla\textsubscript{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\textsubscript{OXA23}, bla\textsubscript{OXA40}, bla\textsubscript{OXA58}, bla\textsubscript{IMP}, bla\textsubscript{VIM}, bla\textsubscript{SIM} and bla\textsubscript{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 \textsuperscript{®} Ready Mix; Sigma, St Louis, MO) and 4.7 μl of DNAase-free water. Thermocycling was conducted in Biometra TGradient Thermocycler as follows: 95°C for 2 minutes; 30 cycles of 95°C for 30 seconds, specific annealing temperature for 30 seconds, 68°C for 1.5 minutes; final step at 68°C 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 (1\textsuperscript{st} 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 \textsuperscript{®} Ready Mix) and 5.7 μl of DNAase-free water. Thermocycling was conducted in Biometra TGradient Thermocycler 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 (Santimaleeworagun et al, 2014). Amplicons were separated by 2% agarose gel electrophoresis.
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 \textit{A. baumannii} isolates using GeneJet Plasmid Miniprep Kit (Thermo Scientific), digested with \textit{EcoRI} (New England BioLabs, Ipswich, MA) and analyzed by 1% agarose gel-electrophoresis.

RESULTS

Identification of carbapenemase genes

Carbapenemase genes, \textit{bla}\textsubscript{OXA23}, \textit{bla}\textsubscript{OXA40}, \textit{bla}\textsubscript{OXA58}, \textit{bla}\textsubscript{VIM}, \textit{bla}\textsubscript{SIM} and \textit{bla}\textsubscript{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 \textit{bla}\textsubscript{OXA23} and \textit{bla}\textsubscript{VIM}, 2 with co-existing \textit{bla}\textsubscript{OXA23} and \textit{bla}\textsubscript{NDM}, and 1 with co-existing \textit{bla}\textsubscript{VIM} and \textit{bla}\textsubscript{NDM}. It is of interest to note that one isolate, CRAB45, contained \textit{bla}\textsubscript{OXA23}, \textit{bla}\textsubscript{VIM} and \textit{bla}\textsubscript{NDM}.

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 \textit{bla}\textsubscript{OXA23}, \textit{bla}\textsubscript{OXA23} + \textit{bla}\textsubscript{VIM}, \textit{bla}\textsubscript{OXA23} + \textit{bla}\textsubscript{NDM}, \textit{bla}\textsubscript{OXA23} + \textit{bla}\textsubscript{VIM} + \textit{bla}\textsubscript{NDM},...
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.

+ blaNDM1, and blaOXA23 + blaVIM2 + blaNDM1 belonged to REP Pattern I, whereas isolates carrying blaVIM2 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 blaOXA23-positive CRAB isolates, whereas plasmid pattern B and C were widespread among CRAB isolates carrying blaOXA23 + blaVIM2 (Table 2). In addition, plasmid pattern C was present in isolates carrying blaOXA23 + blaNDM1, and blaOXA23 + blaVIM2 + blaNDM1. 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 blaVIM-positive CRAB isolates.

**DISCUSSION**

In this study, we found several carbapenemase genes including blaOXA23, blaVIM, and blaNDM1 among clinical A. baumannii isolates. The majority of CRAB isolates contained blaOXA23 in agreement with previous studies in various
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Table 2

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

<table>
<thead>
<tr>
<th>Carbapenemase genotype</th>
<th>No. of isolates</th>
<th>REP-PCR pattern (n)</th>
<th>Plasmid profile pattern (n)</th>
</tr>
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<tbody>
<tr>
<td>bla_{OXA23}</td>
<td>20</td>
<td>I (17)</td>
<td>A (15), D (1), G (1)</td>
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<tr>
<td></td>
<td></td>
<td>III (1)</td>
<td>F (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VII (1)</td>
<td>n/a (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VIII (1)</td>
<td>D (1)</td>
</tr>
<tr>
<td>bla_{VIM}</td>
<td>7</td>
<td>I (2)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>III (1)</td>
<td>n/a (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV (2)</td>
<td>n/a (1), K (1)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>n/a (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VI (1)</td>
<td>I (1)</td>
</tr>
<tr>
<td>bla_{OXA23} + bla_{VIM}</td>
<td>19</td>
<td>I (18)</td>
<td>B (6), C (10), M(1), R (1)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>B (1)</td>
</tr>
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<td>2</td>
<td>I (2)</td>
<td>C (2)</td>
</tr>
<tr>
<td>bla_{VIM} + bla_{NDM}</td>
<td>1</td>
<td>I (1)</td>
<td>J (1)</td>
</tr>
<tr>
<td>bla_{OXA23} + bla_{VIM} + bla_{NDM}</td>
<td>1</td>
<td>I (1)</td>
<td>C (1)</td>
</tr>
<tr>
<td>None</td>
<td>6</td>
<td>IV (2)</td>
<td>H (1), VI (n/a)</td>
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<td>E (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XII (1)</td>
<td>n/a (1)</td>
</tr>
</tbody>
</table>

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 $\text{bla}_{\text{OXA23}}$, 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 $A. \text{baumanni}$ carrying $\text{bla}_{\text{OXA23}}$ ranged from 16-256 $\mu$g/ml, and that the co-existence of different types of carbapenemases in $A. \text{baumanni}$ increased MIC of imipenem to $> 512 \mu$g/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 $\text{bla}_{\text{VIM2}}$ and $\text{bla}_{\text{NDM1}}$ 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.

ACKNOWLEDGEMENTS

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