Original article

The genotypic detection of *bla*$_{VIM-2}$ among clinical carbapenem-resistant *Pseudomonas aeruginosa* isolates from Phramongkutklao Hospital, Thailand

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Abstract:

This study aimed to determine the presence of Verona integron-encoded metallo-β-lactamase-2 (VIM2) gene among clinical Pseudomonas aeruginosa isolates. All studied isolatese were collected from patients admitted at Phramongkutklao Hospital, Bangkok, Thailand in 2009. The included P. aeruginosa isolates for this study were imipenem or meropenem non-susceptible strains according to Clinical and Laboratory Standards Institute. The antimicrobial susceptibility testing was determined using disk diffusion. VIM2-gene detection was assessed by polymerase chain reaction technique. Three hundred and three carbapenem non-susceptible P. aeruginosa isolates (CNS-PA) were included. One hundred and sixty two (53.46%) were detected metalo-betalactamase production. Thirty-eight out of 303 CNS-PA isolates (12.54%) harboring bla$_{VIM2}$ *gene were detected phenotypic production of metalo-betalactamase. Thus, bla*$_{VIM2}$ *gene partially plays a role for carbapenem resistance, however, most studies on CNS-PA isolates did not include study on genes. There should be further investigation on the carbapenem resistance mechanisms.*

Keywords: ● *Carbapenem* ● *Carbapenemase* ● *Minimum inhibitory concentration* ● *Verona integron-encoded metallo-β-lactamase*

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นิพนธ์ต้นฉบับ

การตรวจหายีน *bla*$_{vim-2}$ ใน *Pseudomonas aeruginosa* ที่ดื้อต่อยากลุ่ม carbapenem ที่แยกได้จากโรงพยาบาลพระมงกุฎเกล้า ประเทศไทย

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บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์ เพื่อตรวจหา Verona integron-encoded metallo-β-lactamase-2 (VIM2) gene จากเชื้อ Pseudomonas aeruginosa ที่แยกได้จากผู้ป่วยในโรงพยาบาลพระมงกุฎเกล้า ประเทศไทย ในปี พ.ศ. 2552 โดยทุกสายพันธุ์เป็นเชื้อที่ไม่ไวต่อยา imipenem หรือ meropenem จากการทดสอบด้วยวิธี disk diffusion และแปลผลตามมาตรฐาน CLSI ได้ทำการทดสอบหายีน VIM-2 โดยใช้วิธี polymerase chain reaction technique (PCR) ผลการศึกษา พบว่าเชื้อ P. aeruginosa ที่ไม่ไวต่อยากลุ่ม carbapenem (CNS-PA) มีจำนวนทั้งหมด 303 สายพันธุ์ โดยพบมีการสร้าง metalo-betalactamase จำนวน162 สายพันธุ์ (ร้อยละ 53.46) และตรวจพบมียีน bla$_{VIM-2}$ จำนวน 38 สายพันธุ์ คิดเป็นอัตราร้อยละ 12.54 (38/303) โดยพบว่าทุกสายพันธุ์ที่มียีน bla$_{VIM-2}$ ตรวจพบว่ามีการสร้าง metalo-betalactamase สรุปได้ว่ายีน bla$_{VIM-2}$ น่าจะเป็นกลไกหนึ่งที่มีบทบาทในการทำให้เกิดการ ดื้อยากลุ่ม carbapenem ในครั้งนี้ อย่างไรก็ตามการศึกษาเชื้อ CNS-PA ส่วนใหญ่ยังไม่ค่อยมีการศึกษาเกี่ยวกับยีนมากนักจึงควรมีการศึกษาเพิ่มเติมต่อไปในเรื่องกลไกการดื้อยากลุ่ม carbapenem ของเชื้อกลุ่มนี้

คำสำคัญ: ● *Carbapenem* ● *Carbapenemase* ● *Minimum inhibitory concentration*
● *Verona integron-encoded metallo-β-lactamase*

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Introduction

Pseudomonas aeruginosa, a Gram-negative bacteria, is a major cause of either acute or chronic infections, especially in patients who have an immunocompromised status or chronic airway diseases and need admission to intensive care unit. *P. aeruginosa* can cause various symptoms in many organs/systems including the upper and lower respiratory tract, urinary tract, bloodstream, skin and soft tissue, bone and joint, and eyes[1]. The high impact of *P. aeruginosa* infection on morbidity and mortality might be explained by the patient's immune status, severity of acute illness in clinical presentation, virulent factors of pathogens, and its antibiotic resistance[2-4]. Particularly for carbapenemase carrying *P. aeruginosa*, the bacteremic patients with Verona Integron-encoded Metallo-β-lactamase (VIM) significantly showed 28-day all-cause mortality rate (42.5%) higher than those with negative-VIM *P. aeruginosa* isolates (19.6%)[5].

At present, *P. aeruginosa* has increased its resistance to many antimicrobials agents. Multi-mechanism of drug resistances has been reported for *P. aeruginosa* including enzyme destroying or modifying antibiotic structure, loss of porin, efflux pump, and alteration of target drug[6].

Metallo-β-lactamases (MBL), carbapenemase enzyme classified in Amber class B, were mostly found in *P. aeruginosa* expressing carbapenem resistance. The two previous studies in Thailand reported the presence of VIM-2 in *P. aeruginosa*[7,8]. However, they could not find bla_{VIM2} gene in carbapenem-resistant *P. aeruginosa* isolates[9]. Thus, the three previous studies in Thailand have diversely reported VIM-2 gene in *P. aeruginosa*. Herein, our study determined the presence of the VIM-2 genes in clinical *P. aeruginosa* isolates to understand the gene being responsible for carbapenem resistance.

Materials and methods

The studied strains

The studied *P. aeruginosa* were obtained from clinical specimens of patients admitted at Phramongkutklao Hospital, Bangkok, Thailand in 2009. This study was approved by the Ethical Review Committee of the Royal Thai Army Medical Department (approval no. S019h/52).

The *P. aeruginosa* isolates were cultured on Trypticase Soy Agar (TSA) at 37°C and were then identified from colony characteristics and standard biochemical testing. The included carbapenem non-susceptible *P. aeruginosa* isolates (CNS-PA) for this study were non-repeated clinical strains exhibiting either imipenem or meropenem resistance by Kirby-Bauer disk diffusion test according to Clinical and Laboratory Standards Institute (CLSI). The pure *P. aeruginosa* isolates were kept at -70°C until tested.

Determination of antibacterial susceptibility

The phenotypic activity of carbapenems (imipenem and meropenem) against CNS-PA isolates was determined as minimum inhibitory concentration (MIC) using the E-test (Biomerieux, MA, USA). The culture conditions such as agar, temperature, and time to susceptibility interpretation were followed by CLSI. The *P. aeruginosa* ATCC 27823 as the reference strain was used for the quality control of antimicrobial testing.

Phenotypic detection of metallo-beta-lactamase producing strains

We performed the phenotypic detection of metallo-beta-lactamase (MBL) producing strains by ethylenediaminetetraacetic acid (EDTA) disk synergy test[12]. A McFarland no. 0.5 turbidity of studied CNS-PA isolates was inoculated on a Mueller-Hinton agar plate. After drying, 10 μg of imipenem disk, 10 μg of meropenem disk, and a blank disk impregnated with 1.5 mg were placed 10 mm apart from edge to edge. After overnight incubation, the presence of an enlarged zone of inhibition of imipenem or meropenem disk nearest EDTA disk was interpreted as MBL-producing CNS-PA.

Detection of *bla*$_{VIM-2}$

VIM-2-gene detection was assessed by polymerase chain reaction (PCR) technique. The regrowth *P. aeruginosa* isolates on TSA was used. The DNA from cell of *P. aeruginosa* isolates was extracted by boiling the cell in 1 mL of deionized water for 15 minutes in a water bath, and then centrifuging for 15 minutes at 3,000 rpm. The *bla*$_{VIM2}$ gene was detected by PCR, using specific a couple of primers (VIM-2 F; 5'-ATG TTC AAA CTT TTG AGT AAG -3' and VIM-2 R; 5'- CTA CTC AAC GAC TGA GCG-3') [13].

The 25 µL PCR mixture consisted of 1 µL of DNA extraction, 1 µL of each primer (20 µM), forward and reward, 0.5 µL of dNTP (10 mM), 0.75 µL of $MgCl_2$ (50 mM), 2.5 µL of Buffer (10 X), 0.10 µL of Tag polymerase (5U), and 18.15 µL of DNase-free water.

The PCR conditions were firstly denaturation at 94ºC for 5 minutes, followed by 30 cycles of 94ºC for 60 seconds, annealing temperature at 55 ºC for 60 seconds, extension period of 72ºC for 60 seconds, and lastly final extension at 72ºC for 5 minutes. All PCR procedures were conducted in Thermocycler (Alpha Cycler4, PCRmax, UK). The PCR amplicons were visualized by electrophoresis on 1% agarose gels stained with ethidium bromide. The target gene of *bla*$_{VIM2}$ in the studied isolates was compared with the reference strain having by size of 801 base-pair, as shown in Figure 1.

Results

During 12-month study period, 303 CNS-PA isolates were included. Among 303 CNS-PA isolates, they showed universally non-susceptible to imipenem (intermediate 2.6% and resistant 97.4%) but 53 out of 303 CNS-PA isolates (17.5%) remained susceptible to meropenem.

With 303 CNS-PA isolates, the presence of MBL production revealed 162 isolates detected by disk synergy test. Among 162 MBL positive isolates, the *bla*$_{VIM2}$ gene in the 38 studied *P. aeruginosa* isolates (12.5%) was detected by PCR. The MIC range (MIC50) for imipenem and meropenem were: 4->32 µg/mL (16 µg/mL) and 2->32 µg/mL (16 µg/mL), respectively.

Discussion

Among studied 162 clinical *P. aeruginosa* isolates with MBL production, 38 out of them carried only *bla*$_{VIM-2}$. Our finding was similar to a previous study conducted in Thailand revealing the *P. aeruginosa* carrying *bla*$_{VIM-2}$ [7,8], and our study with *P. aeruginosa* isolates collected in 2009. This finding was consistent with earlier findings from our setting that Pungcharoenkijkul et al reported *P. aeruginosa* isolate carried the *bla*$_{VIM}$ metallo-beta-lactamase gene[14]. However, the other carbapenemase genes such as IMP-1 and IMP-14 were also reported in Thailand[7-9]. Thus, the remaining strains with no *bla*$_{VIM-2}$ but positive for MBL production must be further studied in the other types of carbapenemase.

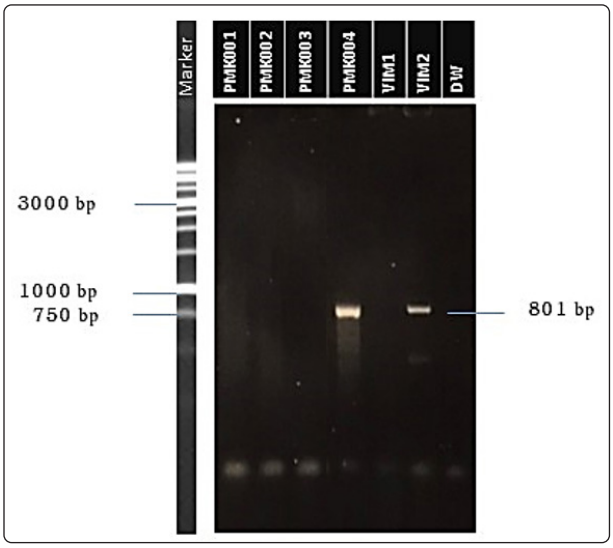

Figure 1 PCR detection of the presence of VIM-2 gene in *Pseudomonas aeruginosa* isolate (PMK004) at 801 bp and the absence VIM-2 gene in three isolates (PMK001-003). Ladder molecular size markers (size (bp) are indicated on the left margin; DW, distilled water as a negative control; VIM as a positive control; PMK001-004, test samples.

Moreover, our findings and previous studies also confirmed that *bla*$_{KPC}$ or *bla*$_{NDM}$ in *P. aeruginosa* were not reported in Thailand. These carbapenemase genes have been scarcely and recently reported in Singapore[10] and Bahrain[11] for NDM and Columbia[15] and Canada[16] for KPC. However, the emerging of *bla*$_{KPC}$ or *bla*$_{NDM}$ among clinical *P. aeruginosa* isolates has to be closely monitored.

Nevertheless, the other mechanisms of carbapenem resistance were not determined in our study. Several previous reports in Thailand have shown the depletion of OprD porin protein among clinical CR-PA isolates[8,17]. The isolates were resistant to imipenem but susceptible to meropenem, producing less OprD protein 3-5 times comparing with the *P. aeruginosa* wild type strains. Moreover, the isolates without OprD porin production showed both of imipenem and meropenem resistance[17].

The mechanism of resistance in *P. aeruginosa* is not only porin losing but also efflux pump. Khuntayaporn et al. revealed that the decreased expression of OprD was a predominant mechanism followed by increased expression of efflux pump system such as MexAB-OprM and MexXY. Whereas, IMP-1, IMP-14 and VIM-2 as MBL were detected in one third of *P. aeruginosa* isolates[8].

At present, many beta-lactamase inhibitors e.g. avibactam or vaborbactam could suppresse the enzyme activity categorized in class A, class C, and some enzymes in class D beta-lactamases according to Amber classification, whereas there is no effect of inhibitor on class B-enzyme (MBL). Thus, our studied strains harbouring *bla*$_{VIM2}$ could not inhibit by such beta-lactamase inhibitors. This is a challenging treatment for investigating a novel β-lactamase inhibitor against MBL producing pathogens[18].

In conclusion, we revealed the *bla*$_{VIM2}$ gene harbouring in clinical *P. aeruginosa* isolates. However, the other mechanisms and carbapenemase genes other than *bla*$_{VIM2}$ will continue to be closely monitored.

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