STUDY OF CARBAPENEM-RESISTANT *PSEUDOMONAS AERUGINOSA* (CRPA) BY PHENOTYPIC AND MOLECULAR APPROACHES

JAKKRIT KHAMPHAKUL

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfilment of the Requirements
for the Master of Science in Medical Technology
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by Jakkrit Khamphakul

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Medical Technology of Naresuan University

Oral Defense Committee

…….Chair

(Associate Professor Panan Ratthawongjirakul, Ph.D.)

…….Advisor

(Assistant Professor Noppadon Jumroon, Ph.D.)

…….Co-Advisor

(Associate Professor Pitak Santanirand, Ph.D.

…….Internal Examiner

(Assistant Professor Worawat Songjang, Ph.D.)

Approved

……...

(Assistant Professor Chommanad Intajamornrak, Ph.D.)

Head, Department of Linguistics, Folklore, Philosophy and Religion, Faculty of Humanities

Acting Dean of the Graduate School

Title	STUDY OF CARBAPENEM-RESISTANT PSEUDOMONAS AERUGINOSA (CRPA) BY PHENOTYPIC AND MOLECULAR APPROACHES
Author	Jakkrit Khamphakul
Advisor	Assistant Professor Noppadon Jumroon, Ph.D.
Co-Advisor	Associate Professor Pitak Santanirand, Ph.D.
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ABSTRACT

Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) is a prominent healthcare-associated pathogen causing public health concerns worldwide. Different resistant mechanisms, either carbapenemase-producing or non-carbapenemase-producing, such as porin loss and efflux pump, may require different treatment approaches. In addition, the distribution of strains and resistant genes varies among global regions. A limited number of reports regarding this issue have been published in Thailand. Therefore, this study aimed to establish the information on the bacterial strains, resistant genes, and their correlation using the molecular approaches, as well as establishing the efficacy of the modified Carbapenem Inactivation Method (mCIM) for detecting carbapenemase-producing strain. In this study, 164 CRPA isolates were tested for carbapenemase genes by the direct flow chip kits covering 17 carbapenem-resistant genes. Of these isolates, five families of carbapenemase genes, including *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{GES}$, and *bla*$_{SIM}$, were detected in 83 isolates, while 81 were negative for carbapenemase genes. The *bla*$_{IMP}$ was the most prevalent, followed by *bla*$_{VIM}$ and *bla*$_{NDM}$. Only 68/83 (83.13%) of carbapenemase gene-positive isolates were positive by the mCIM assay. Of the fifteen mCIM-negative isolates, six (40%) and four (26.67%) isolates contained *bla*$_{VIM-2}$ and *bla*$_{IMP-62}$, respectively; however, one

was found to co-exist with two genes. Five (33.33%) carried *bla*$_{GES-5}$, and one isolate with *bla*$_{VIM-63}$.

A total of 53 sequence types (STs) and four unmatched STs of CRPA were found in this study. Of these 53 STs, only twelve (22.64%) were associated with carbapenemase genes. The ST235, ST357, and ST964 were the top three most common sequence types containing carbapenemase genes, while ST244 was the most common non-carbapenemase-producing CRPA. Although the resistant genes found in these STs varied, each ST had predominant genes. The *bla*$_{IMP}$ was dominant in ST235, while *bla*$_{VIM}$ and a combination of *bla*$_{NDM}$ plus *bla*$_{IMP}$ were dominant in ST357 and ST964, respectively. Among 83 carbapenemase-producing *P. aeruginosa* (CPPA), 81.93% carried a single resistant gene. However, 66.67% of ST964 contained a combination of *bla*$_{IMP}$ and *bla*$_{NDM}$. Interestingly, four carbapenemase genes were detected in an isolate of ST235.

The antimicrobial susceptibility profile of CRPA revealed that of 20 tested antibiotics, the MIC50 of only four antibiotics, including amikacin, netilmicin, cefiderocol, and cefepime-zidebactam, was in the susceptible category. Only cefepime-zidebactam showed the MIC90 within susceptible breakpoint, while the MIC90 of cefiderocol was in the intermediate range. Interestingly, the MIC50 and MIC90 of colistin remained in the intermediate breakpoint. The other fifteen antibiotics showed no effect against CRPA. Among the three most common STs of CPPA, ST357, in which most of the isolates contained *bla*$_{VIM}$, had the strongest resistant activity. Only amikacin had the MIC90 in the susceptible range, and cefepime-zidebactem and netilmicin remained at the intermediate level.

The data of this project revealed the distribution of CRPA strains, the association of STs and carbapenemase genes, and the antimicrobial susceptibility profiles. The results also indicated that minimal antibiotic choices are good candidates for treating CRPA infection.

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Jakkrit Khamphakul

LIST OF CONTENTS

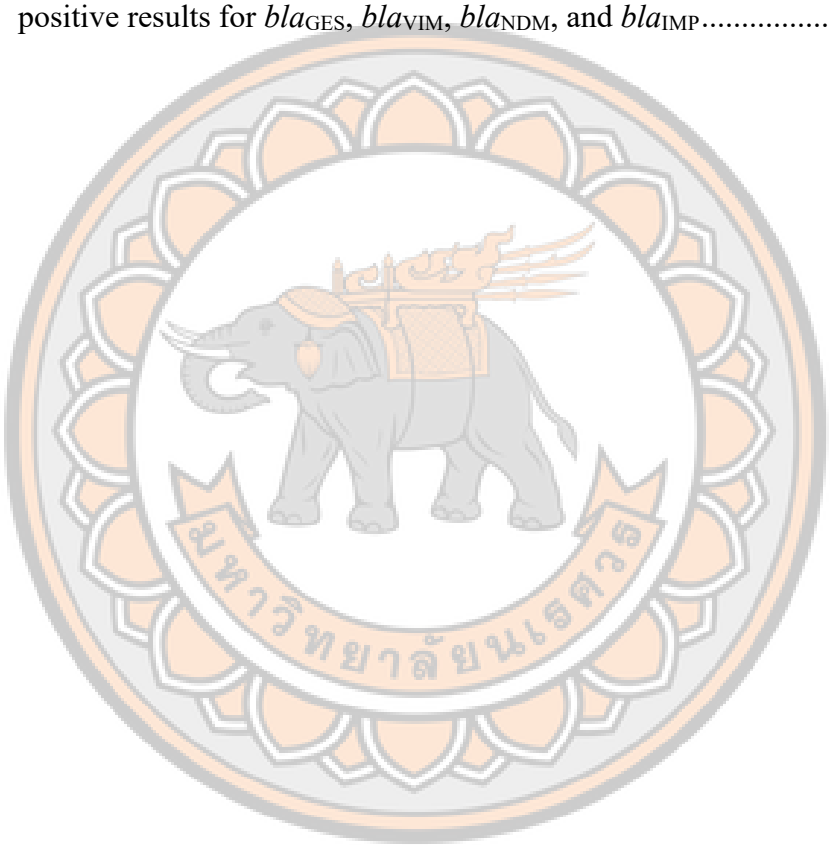
Page

LIST OF TABLES

Page

LIST OF FIGURES

Page



CHAPTER I

INTRODUCTION

Background and significance of the study

Pseudomonas aeruginosa, the well-known organism that causes hospital-acquired infections globally (1). Infections cause high-level resistance expression like multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug-resistant (PDR) strains and are associated with prolonged hospital stays, increased morbidity, higher treatment costs, and a rise in mortality rate (1,2). These organisms can receive genetic material, such as plasmids and mobile genetic elements carrying antimicrobial resistance genes (1,3). One of the most important resistance mechanisms in *P. aeruginosa* produces metallo-β-lactamases (MBLs) (3,4). MBL-ambler class B β-lactamases require zinc ions to hydrolyse carbapenem agents, thereby limiting treatment options (4). Various families of MBLs, including bla_{IMP}, bla_{VIM}, and bla_{NDM}, have been reported worldwide, with multiple resistance patterns (3,5). The distribution of these resistance genes often varies by geographic region (3). Identifying resistance genes in carbapenem-resistant *P. aeruginosa* (CRPA), along with their strain types and antimicrobial susceptibility patterns, is essential for understanding these infections and guiding appropriate antimicrobial therapy (3,5).

Purposes of the study

The research aims to investigate the molecular drug resistance mechanism of carbapenem-resistant *P. aeruginosa* (CRPA).

Statement of the problems

The results of this research study can be applied as a guideline to prevent the spread of CRPA and to predict drug resistance of various types of pathogens leading to the correct treatment of patients with antibiotics.

Scope of the study

The study will test with CRPA strains obtained through routine testing in the laboratory of microbiology, the pathology department, the faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand, from February 2019 to June 2021.

Hypotheses of the study

Antimicrobial susceptibility patterns of carbapenem-resistant *P. aeruginosa* (CRPA) have different drug resistance genes, which were obtained from studies at the molecular level using the multiplex PCR method.

CHAPTER II
LITERATURE REVIEW

Related Works and Studies

Pseudomonas aeruginosa, a gram-negative bacillus, non-fermenting bacillus, is well recognised for causing high hospital-acquired infections, especially in the urinary tract, lungs (pneumonia), and abdominal infection (6,7). These infections can lead to high mortality rates associated with septicemia, ranging from 3% to 38.9% (6). Previous studies have revealed high resistance rates of *P. aeruginosa* to various antibiotics, such as cefepime (FEP), ceftazidime (CAZ), colistin (COL), aminoglycosides group-gentamicin (GM), amikacin (AN), and netilmicin (NET), and ciprofloxacin (CIP), and imipenem (IMP) (7,8), which decrease treatment outcomes. Definition of multidrug-resistant (MDR) *P. aeruginosa*, resistance to at least three classes of antimicrobial agents, while extensively drug-resistant (XDR), organisms are susceptible to only one or two classes of antimicrobial groups (2). These pathogens are associated with prolonged admission in hospital, increased complications of treatment, higher costs, and promote mortality rates up to 50 to 60% (2,6).

The prevalence of MDR-PA and XDR-PA is significantly increasing worldwide, obviously in intensive care units (ICUs) (7,9). For example, previous studies have reported rates of drug-resistant *P. aeruginosa* in ICU settings in different regions show an increase, including Asia and the United States (8,9). This organism is an opportunistic pathogen that can spread through endogenous flora and environmental sources (6). Factors such as adherence to infection control practices by healthcare workers and environmental hygiene significantly influence the incidence of hospital-acquired infections, especially in high-risk areas like ICUs (6,7). Molecular typing is an important tool for understanding the epidemiology and transmission of infectious diseases. At the local level, it enables tracking of outbreaks and transmission chains, while at the global level, it facilitates monitoring of the spread of specific clones over time (10,11). Pseudomonas aeruginosa exhibits a non-clonal epidemic population structure, characterised by high genetic diversity with the emergence of successful epidemic clones (10). One widely used method for strain typing is multilocus sequence typing (MLST), which analyses internal fragments of

housekeeping genes, including *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*, to assign sequence types and distinguish genetic variation arising from mutation or recombination (11).

P. aeruginosa can acquire foreign genetic material, such as plasmids and other mobile genetic elements, contributing to the development of antimicrobial resistance (7). The organism commonly produces β-lactamases, including extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs), which confer resistance to β-lactam antibiotics (4,13). MBLs belong to Ambler class B β-lactamases and require metal ions (usually zinc) as cofactors to hydrolyse carbapenems; important types include *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{NDM}$, which are distributed globally (4). Carbapenem-resistant

P. aeruginosa (CRPA) demonstrates significantly higher levels of resistance compared to susceptible strains and often shows cross-resistance to multiple antibiotic classes (4,7). Detection of MBL-producing organisms can be challenging, and treatment of severe infections often requires combination therapy or last-resort antibiotics such as colistin, depending on local susceptibility patterns (7). B-lactamases are classified into four molecular classes (A-D) based on amino acid sequence homology according to the Ambler classification system, while the Bush–Jacoby classification categorizes them based on functional characteristics, including substrate and inhibitor profiles (12). Historically, *bla*$_{TEM}$-type β-lactamases were among the first plasmid-mediated enzymes identified in *Escherichia coli*, contributing to resistance against β-lactam antibiotics. *Bla*$_{SHV}$-type enzymes, also belonging to class A, are commonly associated with *E. coli and Klebsiella pneumoniae* and are frequently implicated in hospital-acquired infections (12). ESBLs, which arise from mutations in genes such as *bla*$_{TEM}$ and *bla*$_{SHV}$, are capable of hydrolysing extended-spectrum cephalosporins, including cefotaxime, ceftriaxone, and ceftazidime, as well as aztreonam (13).

Class A Serine Carbapenemases

The evolution of β-lactamases enzymes has led to the development of non-metallo β-lactamases, which can break down a wide range of antibiotics, including carbapenems, cephalosporins, penicillin, and aztreonam. This shift from penicillins and cephalosporins to the carbapenem group. The *Klebsiella pneumoniae* carbapenemase (KPC) enzymes are the most well-recognised and well-explained in class A serine carbapenemases. As described in *K. pneumoniae*, KPC is commonly found in various members of the Enterobacteriaceae family. Organisms that carry bla_{KPC} genes can resist all β-lactam agents except carbapenems and monobactams. These genes can be expressed on mobile genetics such as transposons (ex, Tn4401b) and other plasmid types (including IncFII, IncL/M, and IncN). Bacteria that express bla_{KPC} genes are highly multi-drug resistant (MDR) because they are often resistant to many antibiotics, including a drug in a group of quinolones and aminoglycosides. As a result, these bacteria are difficult to manage and threaten public health (4,14).

Class B Beta-Lactamases

Class B β-lactamases, as well known in metallo-β-lactamases (MBLs), are commonly found in *P. aeruginosa* and *Acinetobacter spp.*, and are increasingly reported in Enterobacterales, including *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter spp.* The most clinically important enzymes of the MBL family include Verona integron-encoded metallo-β-lactamase (VIM), imipenemase (IMP), and New Delhi metallo-β-lactamase (NDM). These enzymes are frequently correlated with mobile genetic elements, including integrons, plasmids, and transposons, facilitating their horizontal dissemination (3,15). MBLs can hydrolyse a broad range of β-lactam antibiotics, such as penicillins, cephalosporins, and carbapenems; they do not hydrolyse monobactams such as aztreonam. Importantly, MBLs are not inhibited by conventional β-lactamase inhibitors such as clavulanic acid, tazobactam, or sulbactam. In contrast to serine β-lactamases such as bla_{KPC}, MBLs require supplements like zinc ions at their active site for enzymatic activity (19). The dissemination of MBL genes, particularly bla_{NDM}, has been widely reported among Enterobacterales and non-fermenting bacteria, contributing significantly to global antimicrobial resistance.

Numerous *bla*$_{NDM}$ variants have been identified and are associated with high-risk epidemic clones such as *K. pneumoniae* ST11 and ST147, as well as *E. coli* ST131 (3). Similarly, *bla*$_{IMP}$-type MBLs have been frequently reported in Asia, particularly in Japan, and are associated with plasmid-mediated spread. Unlike previously described, MBLs generally confer high-level resistance to carbapenems, including both imipenem and meropenem, although variability in susceptibility may occur depending on expression levels, permeability, and additional resistance mechanisms (15).

Class C, Serine-Based Cephalosporinases

AmpC β-lactamases are chromosomally encoded cephalosporinases commonly found in members of SPICE organisms, including *Serratia spp.*, *Pseudomonas aeruginosa*, *Providencia spp.*, indole-positive *Proteus sp.* (*P. vulgaris*), *Citrobacter spp.*, and *Enterobacter spp.* These enzymes refer to resistance to a broad range of β-lactam antibiotics, including penicillins, various third-generation cephalosporins (such as cefotaxime (CTX) and ceftriaxone (CRO), and the cephamycin group (including cefoxitin and cefotetan), and are not inhibited by traditional β-lactamase inhibitors such as clavulanic acid, tazobactam, or sulbactam. In many Enterobacterales, *ampC* expression is typically low but can be strongly induced in the presence of certain β-lactam antibiotics. This inducible expression is regulated by a complex system involving cell wall recycling. Accumulation of cell wall degradation products (muropeptides) activates the transcriptional regulator AmpR, leading to increased *ampC* expression. The cytosolic amidase AmpD plays a key role in repressing *ampC* expression by degrading these muropeptides; therefore, mutations in the *ampD* gene result in constitutive overproduction of AmpC and increased resistance. Additional regulatory components, including AmpR and AmpG, also influence *ampC* expression, although mutations in these genes are less commonly involved in derepression. Furthermore, high-level resistance is often enhanced by additional mechanisms such as reduced outer membrane permeability (porin loss) and increased efflux pump activity, which together contribute to multidrug resistance phenotypes (16).

Class D Serine Oxacillinases

Class D β-lactamases, also known as oxacillinases (OXA-type enzymes), have emerged as important contributors to antimicrobial resistance in healthcare settings. These enzymes comprise a diverse group of *bla*$_{OXA}$ variants, some of which possess carbapenem-hydrolysing activity, although the level of activity varies significantly among different enzymes. In general, *bla*$_{OXA}$-type β-lactamases are poorly inhibited by traditional β-lactamase inhibitors such as clavulanic acid, tazobactam, and sulbactam, although variability in inhibition profiles has been reported among specific variants (17). Carbapenem-hydrolysing class D β-lactamases are most commonly associated with *Acinetobacter spp.*, but have also been increasingly identified in Enterobacterales, particularly *Klebsiella pneumoniae* (17,18). Among these, *bla*$_{OXA-48}$ is one of the most clinically significant carbapenemases and was first identified in 2004 in a multidrug-resistant *K. pneumoniae* isolate (18). *bla*$_{OXA-48}$ and its variants typically exhibit weak hydrolytic activity against extended-spectrum cephalosporins but are capable of hydrolysing penicillins and, to a lesser extent, carbapenems. The clinical impact of *bla*$_{OXA-48}$-producing organisms is often enhanced by the co-production of other β-lactamases, such as extended-spectrum β-lactamases (e.g., *bla*$_{SHV}$, *bla*$_{TEM}$), as well as additional resistance mechanisms, including decreased outer membrane permeability. These combined mechanisms can result in high-level resistance and limited therapeutic options. OXA-type carbapenemases have now been reported worldwide and are widely disseminated among different members of the Enterobacterales, contributing significantly to the global spread of carbapenem resistance (17,18). Early detection, appropriate antimicrobial stewardship, and infection control measures are essential to limit the spread of these resistant organisms and improve clinical outcomes.

Multiplex PCR and DNA microarray technologies are advanced molecular tools used for the detection of antimicrobial resistance genes in clinical microbiology laboratories. These methods allow the simultaneous detection of multiple genetic targets, enabling rapid and comprehensive analysis of bacterial isolates. They provide valuable information for guiding clinical decision-making and for surveillance of antimicrobial resistance patterns. These techniques can be applied to bacterial cultures

as well as directly to clinical specimens, depending on the assay design, and are capable of producing accurate and clinically relevant results. DNA microarray technology, in particular, has been widely utilised for the detection and characterisation of resistance determinants, including genes encoding carbapenemases and extended-spectrum β-lactamases (ESBLs) in organisms such as Enterobacterales, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, as well as methicillin resistance genes in *Staphylococcus aureus*. Both custom-designed and commercially available platforms have been developed, offering flexibility for research and diagnostic applications. These technologies play an important role in improving the detection of resistance mechanisms and enhancing the monitoring and control of antimicrobial resistance in clinical settings (19).

The modified carbapenem inactivation method (mCIM)

The modified carbapenem inactivation method (mCIM) is widely used for the phenotypic detection of carbapenemase-producing *Pseudomonas aeruginosa* according to Clinical and Laboratory Standards Institute (CLSI) guidelines (20). This method demonstrates high sensitivity and specificity for detecting carbapenemase activity and shows good correlation with molecular methods such as PCR (20,21). However, mCIM may fail to detect certain carbapenemases, particularly Guiana extended-spectrum (GES) enzymes, leading to false-negative results (21). To address this limitation, modifications such as extended incubation time, increased inoculum (double inoculum), or a combination of both have been proposed to improve detection performance (21). Phenotypic methods such as double-disk synergy tests and combined disk tests have also been developed for the detection of metallo-β-lactamase (MBL) production in carbapenem-resistant *P. aeruginosa* (CRPA) (22). The use of mCIM and EDTA-modified CIM (eCIM) is important in clinical laboratories for distinguishing carbapenemase-producing isolates and guiding appropriate antimicrobial therapy (20).

AMR Direct Flow Chip

The AMR Direct Flow Chip (Máster Diagnóstica, Granada, Spain) is a commercially available DNA microarray test that can simultaneously detect multiple antibiotic resistance genes. It starts with isolated colonies and has been approved for clinical use in the European Economic Area (CE IVD), meeting regulatory standards for diagnostic tests performed in vitro. This assay provides accurate detection of resistance genes in Gram-positive and Gram-negative bacteria. It has shown both 100% sensitivity and specificity for all target genes, meaning it correctly identifies the presence and absence of genes in samples. However, the test's performance can vary due to factors like the quality of the material and sample preparation procedure. The AMR Direct Flow Chip Kit uses the principle of multiplex PCR with biotinylated primers to amplify DNA products. Afterward, it automatically identifies resistance genes by reverse hybridization approach on a membrane using specific probes. This machine is able to detect a total of 20 known resistance genes in both Gram-negative bacteria and Gram-positive cocci. (23,28)

Table 1 The resistance genes detected from the AMR Direct Flow Chip Kit.

Class of gene	Gene	Variant
Class A carbapenemase	*bla*$_{GES}$	1-26
	bla$_{SME}$	1-5
	bla$_{KPC}$	1-23
	bla$_{NMC/IMI}$	1-9
Class B carbapenemase	*bla*$_{SIM}$	
	bla$_{GIM}$	1,2
	bla$_{SPM}$	
	bla$_{NDM}$	1-16
	bla$_{VIM}$	1-46
	bla$_{IMP}$	1-11, 15, 19-21, 24, 25, 28-30, 40-42, 47
Class D carbapenemase	*bla*$_{OXA23-like}$	Total 14 variants
	bla$_{OXA24-like}$	Total 7 variants
	bla$_{OXA48-like}$	Total 4 variants
	bla$_{OXA51-like}$	Total 67 variants
	bla$_{OXA58-like}$	Total 4 variants
ESBL	*bla*$_{SHV}$	
	bla$_{CTX-M}$	
Vancomycin resistance	*vanA*	
	vanB	
MRSA	*mecA*	

Source: Pérez-Pérez FJ, Hanson ND. J Clin Microbiol. 2002;40(6)

In Thailand, studies have reported a high prevalence of multidrug-resistant (MDR) *Pseudomonas aeruginosa*, with a substantial proportion classified as carbapenem-resistant strains. High levels of resistance to carbapenems, particularly meropenem, have been consistently observed across multiple hospitals (24). A multicenter study conducted in Thailand between 2007 and 2019 identified *bla*$_{IMP}$ and

bla$_{VIM}$ as the predominant metallo-β-lactamase genes among carbapenem-resistant *P. aeruginosa* isolates (24). Molecular epidemiological analysis revealed that sequence type ST235 is the most prevalent clone in Thailand, followed by ST964 and ST111 (24,37). Notably, ST235 has been detected in both MBL-producing and non-MBL-producing isolates, indicating its adaptability and widespread dissemination. Multilocus sequence typing (MLST) has also identified novel sequence types, such as ST3910, highlighting the genetic diversity of *P. aeruginosa* in the region (24). These isolates commonly harbor multiple antimicrobial resistance genes, including *bla*$_{OXA-50}$ and *aph(3')-IIb*, as well as virulence-associated genes such as *fleN* and *waaA*, which may contribute to their persistence and pathogenicity (37).

CHAPTER III

RESEARCH METHODOLOGY

Bacterial isolates

A total of 164 carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) isolates from non-duplicate patients at Ramathibodi Hospital, Bangkok, Thailand, from February 2019 to June 2021 was used. A combination of colony morphology, conventional biochemical test and MALDI-TOF (Bruker, Germany) was used to identify *Pseudomonas aeruginosa*. The isolates resisted at least one routinely tested carbapenem, including doripenem, imipenem and meropenem. The organisms were cultured on a blood agar culture medium and stored in refrigeration-resistant tubes with a skim-milk medium at -80°C until used. Each isolate was sub-cultured onto blood agar and incubated at 35-37°C for 18-24 hours before testing.

Antimicrobial susceptibility testing

The broth microdilution (BMD) method was used for the antimicrobial susceptibility testing (AST) in this study. Two commercial panels, Sensititre THAN2F and THAMDR1F panels (ThermoFisher, USA) were used. Twenty-four agents are included in the panels, each with a different range of minimum inhibition concentration (MIC) defined by the manufacturer described in Table 2. Additionally, ceftolozane/tazobactam, not included in the THAN2F or THAMDR1F panels, was tested using an E-test strip (Liofilchem, Italy)

Table 2 List of antimicrobial agents that are included in Sensitire panels.

Group of antimicrobials	Agent	Range of MIC values (mg/L)
Aminoglycoside	Amikacin	8 to 32
	Netilmicin	8 to 16
	Gentamicin	2 to 8
Cephalosporin	Ceftriaxone	0.5 to 32
	Cefotaxime	1 to 32
	Ceftazidime	1 to 32
	Ceftazidime-avibactam	0.12/4 to 64/4
	Cefepime	1 to 32
	Cefepime-ziderbactam	0.12/0.12 to 64/64
	Cefiderocol	0.12 to 64
Carbapenem	Imipenem	0.5 to 16
	Imipenem-relebactam	0.12/4 to 64/4
	Meropenem	0.5 to 16
	Meropenem-vaborbactam	0.12/8 to 64/8
Quinolones	Levofloxacin	0.06 to 8
	Ciprofloxacin	0.06 to 2
	Deltafloxacin	0.03 to 16
	Gatifloxacin	0.03 to 16
Tigecycline	Tigecycline	0.12 to 16
Sulfonamide	Trimethoprim/sulfamethoxazole	1/19 to 4/76
Beta-lactam	Piperacillin-tazobactam	8/4 to 64/4
Polymyxin	Colistin	1 to 8

Modified carbapenem inactivation method (mCIM)

All the isolates were tested for carbapenemase production by the mCIM, which recommended approach by the Clinical and Laboratory Standards Institute (CLSI) guideline (20). A few colonies of carbapenem-resistant *P. aeruginosa* were suspended in 2 mL of brain–heart infusion (BHI) broth. A meropenem disk was added to the suspension and appropriately mixed to allow the disk to reach the bottom of the BHI tube. In this study, the doripenem was tested simultaneously. The suspension was incubated for 4 hours at 35-37°C. Before completion of incubation, the Muller-Hinton agar plate was prepared by inoculating a 0.5 McFarland turbidity suspension of *Escherichia coli* ATCC 25922 and streak on Mueller–Hinton agar (similar to the disk diffusion method). When complete incubation time, the meropenem disk was removed from the suspension tube and placed onto the surface of the agar. The plate was incubated for 20-24 h at 35-37°C. Results were interpreted according to the CLSI guideline (20). *Klebsiella pneumoniae* ATCC BAA-1705 was used as a positive control, while *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC BAA-1706 were used as negative controls (20).

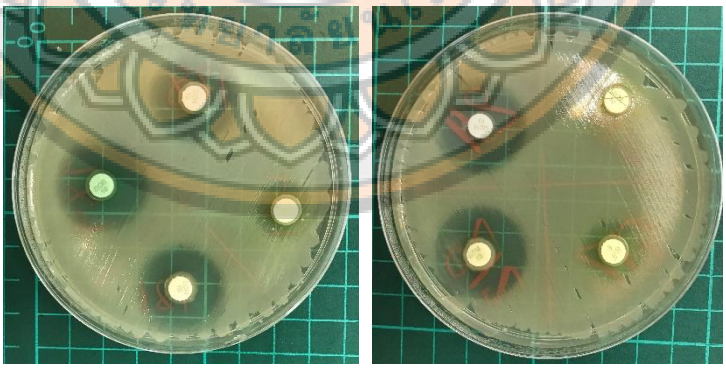
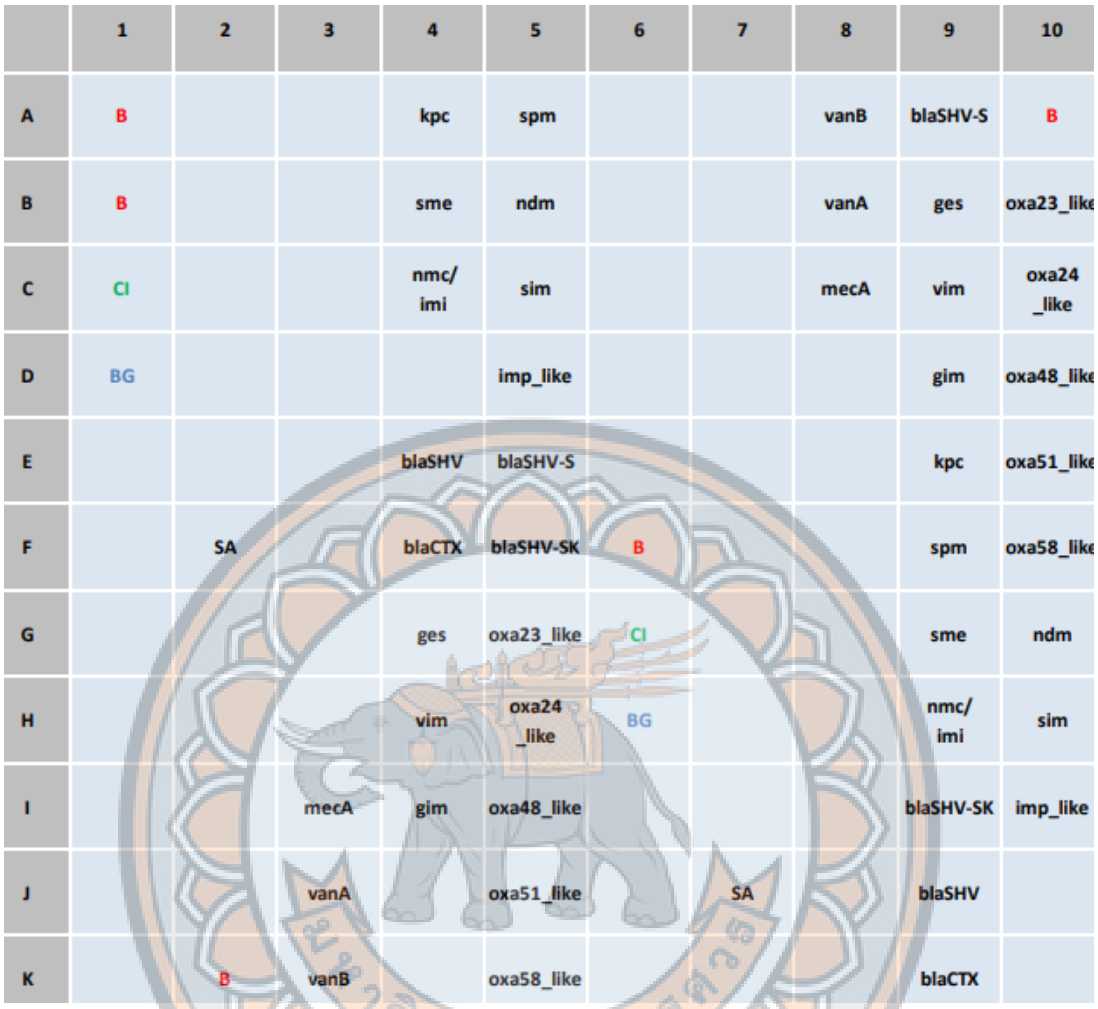


Figure 1 mCIM by meropenem disk (right) and doripenem disk (left) test

Molecular detection of carbapenemase genes by the Direct flow kit

The direct flow kit (Master Diagnostica, Spain) was used for antimicrobial resistance gene detection, like carbapenemase. The principle is based on the DNA target amplified by multiplex PCR and hybridising the PCR products with specific

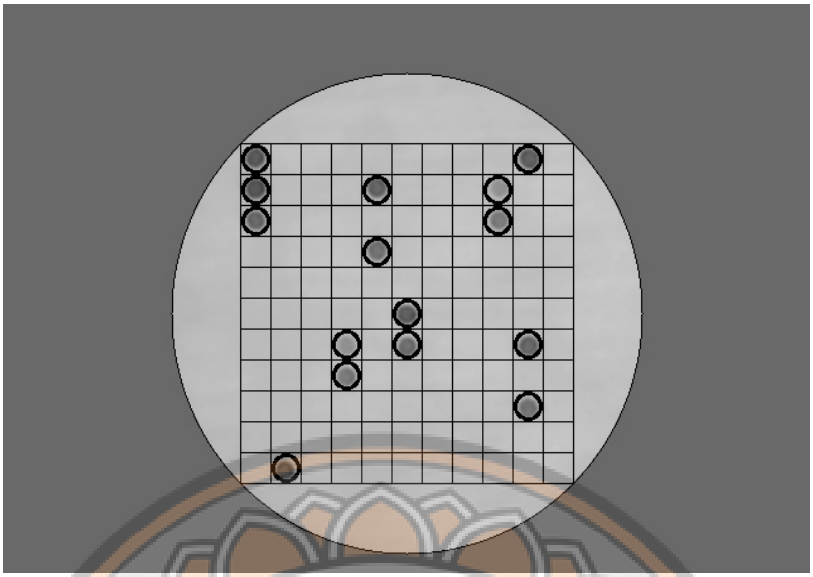
probes that are coated on a membrane (Figure 2). Fifteen carbapenemase genes are shown in Table 1. This study followed the protocol recommended by the manufacturer. Briefly, bacteria were sub-cultured on blood agar medium, and a few colonies were suspended in 500 µl DNase/RNase-free double-distilled water that was included in the kit, used directly for testing without a step of DNA extraction. The suspension was vortexed until homogeneity was obtained. Separate thirty microliters of the suspension and added to the PCR tube containing the lyophilised master mix (Master Diagnostica, Spain). The PCR process was performed in a thermocycler, which had set conditions as follows: one cycle of 10 min at 25°C, then one cycle of 3 min at 95°C, then 40 cycles of 50 sec at 95°C, 45 sec at 55°C and 1 min at 75°C. If we do not run the following process, the PCR tube was kept at 8-10°C before the hybridisation process or at -20°C for further processes. The PCR products were denatured by heating at 95 °C for 8-10 min and quickly cooled in ice for at least 2 min, and these processes occurred in a thermocycler. All hybridisation processes are automatically done by hybriSpot in the HS24 platform. Hybrispot software manages samples, captures images, and reports results.



	1	2	3	4	5	6	7	8	9	10
A	B			kpc	spm			vanB	blaSHV-S	B
B	B			sme	ndm			vanA	ges	oxa23_like
C	CI			nmc/ imi	sim			mecA	vim	oxa24 _like
D	BG				imp_like				gim	oxa48_like
E				blaSHV	blaSHV-S				kpc	oxa51_like
F		SA		blaCTX	blaSHV-SK	B			spm	oxa58_like
G				ges	oxa23_like	CI			sme	ndm
H				vim	oxa24 _like	BG			nmc/ imi	sim
I			mecA	gim	oxa48_like				blaSHV-SK	imp_like
J			vanA		oxa51_like		SA		blaSHV	
K		B	vanB		oxa58_like				blaCTX	

"B": hybridisation control, "CI": Exogenous amplification control, "BG": Endogenous amplification control (fragment human ß-Globin), "X": Specific probes for each resistance marker.

Figure 2 Prob of antimicrobial resistance (AMR) genes coated on the membrane



249 Finished 7/12/2023 10:52:55 AM 7/12/2023 2:44:02 PM AMR POSITIVE

Details of the result:

AMR POSITIVE
Positive sample for:
Carbapenemase GES, Carbapenemase VIM, Carbapenemase NDM, Carbapenemase IMP3 or/and 15 or/and 19_like
Note: Absence of human control DNA.
The sample is negative for the rest of antibiotic resistance genes included in the AMR direct flow chip test.

LOTS => PCR: / Chips: / Reagent: HSH0113-2LF

Figure 3 Picture (top) and result (bottom) of sample No.249, which provides positive results for *bla*$_{GES}$, *bla*$_{VIM}$, *bla*$_{NDM}$, and *bla*$_{IMP}$

Conventional PCR and sequencing of *bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{GES}$ genes

Regarding the limitations of the AMR Direct Flow Chip in detecting variants of bla$_{VIM}$ and bla$_{IMP}$, additional PCR assays targeting these genes were performed for all isolates. Furthermore, isolates that tested negative by mCIM but were positive for bla$_{GES}$ were subjected to confirmatory PCR and sequencing analysis. The primer sequences for bla$_{VIM}$, bla$_{IMP}$, and bla$_{GES}$ are presented in Table 3 (27,28). Conditions for thermocycle set-up start with a denaturation period at 94°C for 10 minutes, and then proceed through 36 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 40 seconds, a following extension step at 72°C for 50 seconds, and a final extension step at 72°C for 5 minutes. The PCR amplicons were processed for sequencing using outsource bidirectional DNA sequencing (Celemic, Republic of Korea). The obtained sequences were analysed using the BLAST program available on

the NCBI database (http://www.ncbi.nlm.nih.gov/blast.cgi) to confirm the identity of β-lactamase genes.

Table 3 Primers of *bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{GES}$ were used in this study.

Gene	Sequence (5' to 3')	Size (bp)
VIM-F	TGTCCGTGATGGTGATGAGT	456
VIM-R	GTGCTTCCGGGTAGTGTTGT	
IMP-F	GGAATAGAGTGGCTTAAYTCTC	382
IMP-R	GGTTTAAYAAAACAACCACC	
GES-F	TCACTCTGCATATGCGTCGG	692
GES-R	ACTTGACCGACAGAGGCAAC	

12. Multilocus sequence typing (MLST)

The CRPA isolates were tested for the multilocus sequence typing (MLST) method. The process of this method begins with DNA extraction by the boiling method at 100 °C for 10 min, followed by a heavy spin in a centrifuge machine at 14000 rpm for 10 min. The supernatant containing DNA was separated into another 2000 microliter sterile tube and kept at -20 °C to preserve DNA. The primers were designed for the listed loci published in the *P. aeruginosa* sequences PUBMLST and procedures. Describes a PCR protocol to amplify the target genes using primers. The reaction mixture is prepared by the commercial (Qiagen, Germany), one μM each forward and reverse primer, and DNAse/RNAse-free water grade, using five microliters of DNA from the extraction. The thermocycler was set up for a protocol that includes 35 cycles of the following steps: denaturation at 96°C for 1 min, primer annealing at 55°C for 1 min, then extension at 72°C for 1 min. PCR amplicons were sent for sequencing (Bionics, Republic of South Korea). Allelic numbers and sequence types (STs) were assigned using the software available via BioEdit version 7.2.5 and PubMLST (http://pubmlst.org/paeruginosa), respectively.

CHAPTER IV

RESULTS

Molecular result

All 164 isolates of Carbapenem-resistant *P. aeruginosa* (CRPA) and eighty-three and eighty-one isolates of Carbapenemase-producing (CP) strain and Non-carbapenemase producing strain (Non-CP) were retrospectively evaluated in this study by molecular method. In the case of Carbapenemase production, all 83 isolates could detect the Carbapenemase genes described previously, but not all isolates could have positive results with the modified Carbapenemase inactivated method (mCIM). Each isolate of CP provides various Carbapenemase genes as show in Table 4 Predominate with 27 isolates of bla_{IMP}, including 19 isolates as $bla_{\text{IMP (3/15/19 group)}}$ was detected by direct flow chip and 8 isolates as $bla_{\text{IMP (non 3/15/19 group)}}$ detected by conventional PCR. Meanwhile, bla_{VIM} sub-predominates with 22 isolates, followed by bla_{NDM} and bla_{GES}, 12 and 6 isolates, respectively.

Table 4 The efficacy of mCIM versus various carbapenemase genes (N=83)

Carbapenemase gene	Number of isolates (%)
bla$_{VIM}$	22 (26.51)
bla$_{IMP\ (3/15/19\ group)}$	19 (22.89)
bla$_{NDM}$	12 (14.46)
bla$_{NDM}$ + *bla*$_{IMP\ (3/15/19\ group)}$	10 (12.05)
bla$_{GES}$	5 (6.02)
bla$_{IMP\ (non\text{-}3/15/19\ group)}$	8 (9.64)
bla$_{VIM}$ + *bla*$_{IMP\ (3/15/19\ group)}$	2 (2.41)
bla$_{VIM}$ + *bla*$_{NDM}$	2 (2.41)
bla$_{SIM}$	1 (1.20)
bla$_{VIM}$ + *bla*$_{GES}$	1 (1.20)
bla$_{VIM}$ + *bla*$_{GES}$ + *bla*$_{NDM}$+ *bla*$_{IMP\ (3/15/19\ group)}$	1 (1.20)

mCIM result

In order to establish the efficacy of mCIM for detecting CPPA, the results of mCIM were compared with molecular testing. Of the 164 isolates, sixty-eight revealed positive results with mCIM, and ninety-six were negative. Additionally, this study investigated another method for mCIM. We used a 10-mg Doripenem disk test instead of a 10-mg Meropenem disk, and we still used the protocol from CLSI guidelines for mCIM testing in the laboratory. All 164 isolates in this study were provided with Meropenem and Doripenem disks with the same result after performing. In these 96 isolates of mCIM-negative, fifteen isolates provided positive results with the carbapenemase genes (Table 5). However, fifteen isolates of the CP strain tested negative in the mCIM test and exhibited results that disagreed with the PCR; the mCIM test was repeated using the mCIM method both via doripenem and meropenem disks. The rate of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the mCIM were 81.93, 100, 100, and 84.38%, respectively.

Table 5 Comparison of Carbapenemase-producing strain and Carbapenemase non-producing strain versus modified Carbapenemase inactivated method (mCIM) result (N=164)

Type of CRPA	Isolates of positive with mCIM	Isolates of negative with mCIM
CPPA	68	15
Non-CPPA	0	81

Among fifteen isolates of mCIM-negative CPPA, 6 (40%), 5 (33.33%) and 3 (20%) carried *bla*$_{VIM}$, *bla*$_{GES}$ and *bla*$_{IMP}$, respectively, and a single isolate containing both *bla*$_{VIM}$ and *bla*$_{IPM}$ (Table 6). Of the fifteen mCIM-negative isolates, *bla*$_{VIM-2}$ was demonstrated in six isolates, with one isolate containing *bla*$_{VIM-2}$ and *bla*$_{IPM-62}$. The majority of these revealed a high level of resistance to all three carbapenems (Table 7). A single isolate had *bla*$_{VIM-63.}$ However, it is worth noting that not all *bla*$_{VIM-2}$ and *bla*$_{VIM-63}$ revealed negative results by mCIM. Approximately 40 and 75% of isolates carrying *bla*$_{VIM-2}$ and *bla*$_{VIM-63}$, respectively, were detected by mCIM (data not shown). The only Imipenemase gene (IMP) type related to a negative mCIM result was *bla*$_{IMP-62}$. All other *bla*$_{IMP}$-containing isolates were mCIM positive. Interestingly, all *bla*$_{GES-5}$ isolates were unable to be detected by mCIM, despite their high-level resistance in 4/5 isolates.

Table 6 The efficacy of mCIM versus various carbapenemase genes

Carbapenemase genes	Number of isolates (N = 83)	
	mCIM positive (N = 68)	mCIM negative (N = 15)
bla$_{VIM}$	16	6
bla$_{IMP}$ (3/15/19 group)	16	3
bla$_{NDM}$	12	0
bla$_{NDM}$ + *bla*$_{IMP}$ (3/15/19 group)	10	0
bla$_{GES}$	0	5
bla$_{IMP}$ (not 3/15/19 group)	8	0
bla$_{VIM}$ + *bla*$_{IMP}$ (3/15/19 group)	1	1
bla$_{VIM}$ + *bla*$_{NDM}$	2	0
bla$_{SIM}$	1	0
bla$_{VIM}$ + *bla*$_{GES}$	1	0
bla$_{VIM}$ + *bla*$_{GES}$ + *bla*$_{NDM}$ + *bla*$_{IMP}$ (3/15/19 group)	1	0

Table 7 Distribution of carbapenemase genes in mCIM-negative carbapenemase-producing (CP) strains of *P. aeruginosa* (N=15)

Carbapenemase genes	Number of isolates	MIC of each isolate (mg/L)		
		Imipenem	Meropenem	Doripenem
*bla*VIM-2	5	≥ 32	≥ 32	≥ 32
		≥ 32	≥ 32	≥ 32
		≥ 32	≥ 32	16
		≥ 32	16	16
		4	8	1
*bla*VIM-63	1	≥ 32	≥ 32	≥ 32
*bla*IMP-62	3	≥ 32	≥ 32	≥ 32
		≥ 32	≥ 32	16
		16	4	4
*bla*IMP-62 + *bla*VIM-2	1	8	16	4
*bla*GES-5	5	≥ 32	≥ 32	≥ 32
		≥ 32	≥ 32	≥ 32
		≥ 32	≥ 32	≥ 32
		≥ 32	≥ 32	≥ 32
		8	2	1

Antimicrobial susceptibility result

In this meticulously conducted research, we thoroughly evaluated 164 isolates of Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA). These isolates, carefully collected from non-duplicate patients admitted to Ramathibodi Hospital in Bangkok, Thailand, exhibited non-susceptibility to at least one Carbapenem agent. The Microbiology laboratory at Ramathibodi Hospital provided comprehensive antimicrobial susceptibility test (AST) profiles for all isolates. These profiles encompassed various drug agents used to combat *P. aeruginosa* infections, including

aminoglycosides (Gentamicin, Netilmicin, and Amikacin), cephalosporins (Ceftazidime and Cefepime), carbapenems (Meropenem, Imipenem, and Doripenem), sulfonamides (Trimethoprim-sulfamethoxazole), fluoroquinolones (Levofloxacin and Ciprofloxacin), polymyxin (Colistin), and Piperacillin-Tazobactam. Additionally, we conducted AST testing for new drugs listed in THAMDR1F (Thermofisher, USA), including Ceftazidime-avibactam, Cefiderocol, Gatifloxacin, Imipenem-relebactam, and Ceftolozane-tazobactam, using E-test (Liofilchem, Italy). This study calculated MIC_{50} and MIC_{90} values, as presented in Table 8. The MIC_{50} values were relatively high for most agents, except the Aminoglycoside group, which included Amikacin and Netilmicin, both of which exhibited $MIC5_0$ values of $\leq$8 mg/L. Colistin displayed an MIC50 value of 2 mg/L, consistent with the MIC90 value, also recorded at 2 mg/L. Notably, Cefiderocol demonstrated a meagre MIC_{50} value of 1 mg/L.

A discernible differentiation in MIC50 and MIC90 values was observed between the CP and Non-CP groups, as demonstrated in Table 9. Amikacin, Cefiderocol, and Colistin exhibit consistent MIC50 values between the CP and Non-CP groups. In contrast, netilmicin demonstrates susceptibility (MIC50) to the organism in the non-CP group but resistance in the CP group. Similarly, Ceftolozane-tazobactam, Ceftazidine-avibactam, and Imipenem-Relebactam provide a significantly lower MIC50 in the non-CP group compared to the high MIC value observed in the CP group.

Table 8 Distribution of MIC_{50} and MIC_{90} of Carbapenem-resistant *P. aeruginosa* (CRPA) (N=164)

Antibiotic	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Amikacin	<=8 to >32	<=8[c]	>32
Gentamicin	<=2 to >8	>8	>8
Netilmicin	<=8 to >16	<=8[c]	>16
Piperacillin/Tazobactam	<=8 to >64	64	>64
Ceftazidime	<=1 to >32	>32	>32
Ceftolozane/Tazobactam[a]	0.5 to >256	>256	>256
Cefiderocol	<=0.12 to >64	1[c]	8
Ceftazidime/Avibactam	0.5 to >64	16	>64
Gatifloxacn	0.12 to >16	16	>16
Imipenem/Relebactam	0.25 to >64	8	>64
Cefepime	<=1 to >32	>32	>32
Doripenem	<=0.5 to >16	16	>16
Imipenem	<=0.5 to >16	>16	>16
Meropenem	<=0.5 to >16	>16	>16
Ciprofloxacin	<=0.06 to >2	>2	>2
Levofloxacin	0.25 to >8	>8	>8
Colistin	<=1 to >8	2[d]	2

[a] MIC value by E-test method

[b] MIC of Colistin from Broth micro-dilution method (BMD) in panel THAN1F

[c] Interpretation: Susceptible

[d] Interpretation: Intermediate

Table 9 Distribution of MIC_{50} and MIC_{90} for Carbapenemase Producing (CP) against Non-Carbapenemase Producing (Non-CP) *P. aeruginosa.*

Antibiotic	Non-CP (N=81)			CP (N=83)		
	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}
Amikacin	<= 8 to >32	<=8[c]	>32	<= 8 to >32	<=8	>32
Gentamicin	<=2 to >8	<=2 [c]	>8	<=2 to >8	>8	>8
Netilmicin	<=8 to >16	<=8 [c]	16	<=8 to >16	>16	>16
Piperacillin/Tazobactam	<=8 to >64	>64	>64	16 to >64	>64	>64
Ceftazidime	<=1 to >32	32	>32	2 to >32	>32	>32
Ceftolozane/Tazobactam [a]	0.5 to >256	2 [c]	>256	1 to >256	>256	>256
Cefiderocol	<=0.12 to >64	1 [c]	4	<=0.12 to >64	4	8
Cefazidime/Avibactam	0.5 to >64	4 [c]	32	0.5 to >64	>64	>64
Gatifloxacn	0.12 to >16	8	>16	0.25 to >16	>16	>16
Imipenem/Relebactam	0.25 to >64	1[c]	8	0.25 to >64	>64	>64
Cefepime	<=1 to >32	16	>32	4 to >32	>32	>32
Doripenem	<=0.5 to >16	4	16	1 to >16	>16	>16
Imipenem	<=0.5 to >16	16	>16	2 to >16	>16	>16
Meropenem	<=0.5 to >16	8	>16	2 to >16	>16	>16
Ciprofloxacin	<=0.06 to >2	1	>2	0.12 to >2	>2	>2
Levofloxacin	0.25 to >8	4	>8	0.25 to >8	>8	>8
Colistin	<=1 to 4	2[d]	2	<=1 to .8	2[d]	2

[a] MIC value by E-test method,

[b] MIC of Colistin from Broth micro-dilution method (BMD) in panel THAN1F

[c] Interpretation: Susceptible,

[d] Interpretation: Intermediate

In this study, we conducted comparisons of the resistance rates for each antimicrobial agent. Percentage of antimicrobial resistance in the presence of Carbapenemase genes within the group of Carbapenemase-producing *P. aeruginosa* (CPPA) isolates. These isolates displayed intermediate or resistant interpretation results for each drug. Moreover, colistin was included in the calculation when the

isolate showed MIC higher than 2 mg/L. The results demonstrated a wide range of resistance rates, varying from 0% to 100%. Most notably, the *bla*$_{IMP}$ and *bla*$_{VIM}$ gene were frequently detected, associated with high resistance rates for many drugs, except for Amikacin, Cefiderocol, and Colistin, which exhibited resistance rates of 31.58%, and 13.64%, 21.05%, and 45.45%, and 10.53% and 27.27%, respectively. Among most populations carrying Carbapenemase genes, such as *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{GES}$, and *bla*$_{NDM}$ in conjunction with *bla*$_{IMP}$, colistin exhibited a lower percent resistance rate than other drug groups. The resistance rates for colistin ranged from 0% to 27.27%. Similarly, Amikacin and Cefiderocol displayed relatively low resistance rates. Specifically, Amikacin exhibited resistance rates of 13.64% in the *bla*$_{VIM}$ group, 31.58% in the *bla*$_{IMP}$ group, 58.33% in the *bla*$_{NDM}$ group, 20% in the *bla*$_{GES}$ group, and 100% in the *bla*$_{NDM}$ with *bla*$_{IMP}$ group. Cefiderocol demonstrated resistance rates ranging from 0% to 75%. Notably, all isolates in the *bla*$_{GES}$ group were susceptible to cefiderocol. Conversely, cefiderocol displayed resistance rates of 21.05%, 45.45%, and 40% in the *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{NDM}$ with *bla*$_{IMP}$ groups, respectively. However, isolates of Carbapenemase-producing *Pseudomonas aeruginosa* (CPPA) carrying the *bla*$_{NDM}$ gene exhibited a relatively high resistance rate to cefiderocol, approximately 75%.

Table 10 The percentage of not susceptible antimicrobial agents compared to the presence of the Carbapenemase gene in Carbapenemase-producing. *Pseudomonas aeruginosa* strains. (N=83)

Carbapenemase genes	Amikacin	Gentamicin	Netilmicin	Piperacillin/ Tazobactam	Ceftazidime	Ceftolozane/ Tazobactam	Cefiderocol	Ceftazidime/ Avibactam	Gatifloxacin	Imipenem/ Relebactam	Cefepime	Doripenem	Imipenem	Meropenem	Ciprofloxacin	Levofloxacin	Colistin [a]
***bla*IMP (*n*=27)**	32	95	79	68	100	100	21	89	89	89	100	100	95	100	95	95	11
***bla*VIM (*n*=22)**	14	100	45	86	82	95	45	77	91	95	73	95	100	100	100	95	27
***bla*NDM (*n*=12)**	58	100	83	100	100	100	75	100	83	100	100	100	100	100	92	92	8
***bla*GES (*n*=5)**	20	80	90	100	40	60	0	0	80	90	50	90	100	90	90	100	0
***bla*NDM+ *bla*IMP (*n*=10)**	100	100	100	100	100	100	40	100	100	100	100	100	100	100	100	100	0
***bla*SIM (*n*=1)**	0	100	100	100	100	100	0	100	100	100	100	100	100	100	100	100	0
***bla*NDM+ *bla*VIM (*n*=2)**	0	100	100	100	100	100	0	100	100	100	100	100	100	100	100	100	0
***bla*GES+ *bla*VIM (*n*=1)**	0	100	100	100	100	100	0	100	100	100	100	100	100	100	100	100	0
***bla*IMP+ *bla*VIM (*n*=2)**	0	100	0	100	100	100	100	100	100	100	100	100	100	100	100	100	0
***bla*GES +*bla*VIM +*bla*IMP +*bla*NDM (*n*=1)**	100	100	100	100	100	100	0	100	100	100	100	100	100	100	100	100	0

Multilocus sequence typing (MLST) result

In a multilocus sequence typing (MLST) study, one hundred sixty-four isolates of Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) were examined. The CRPA typing yielded diverse results of sequencing typing (ST). Table 11 shows the distribution of ST in CRPA. CRPA exhibited a broad spectrum of sequence types (STs), with 53 STs represented. Notably, ST235 was the predominant ST among CRPA isolates, comprising 27 isolates. ST357, ST964, ST244, ST233, and ST3390 followed with 19, 16, 11, 7, and 7 isolates, respectively. ST654, ST708, and ST823 had six isolates each. ST621 and ST1631 exhibited three isolates each within their groups, and there were 18 ST groups with two isolates each, including ST266, ST277, ST698, ST1195, ST1654, ST2634, ST3986, and ST4073. Moreover, numerous CRPAs presented STs which show with only one isolate. (not shown in the table)

Table 11 Distribution of Sequencing typing (ST) of Carbapenem-resistant *P. aeruginosa* (CRPA).

Sequencing typing (ST)	Number of isolates (%)
235	27 (16.46)
357	19 (11.59)
964	16 (9.76)
244	11 (6.71)
233	7 (4.27)
3390	7 (4.27)
654	6 (3.66)
708	6 (3.66)
823	6 (3.66)
260	4 (2.44)
621	3 (1.83)
1631	3 (1.83)
266	2 (1.22)
277	2 (1.22)
698	2 (1.22)
1195	2 (1.22)
1654	2 (1.22)
2634	2 (1.22)
3986	2 (1.22)
4073	2 (1.22)

The distribution of sequence types (STs) was evaluated compared to Carbapenemase genes. Only thirteen groups of STs tested positive for the Carbapenemase-producing isolates. The predominant ST was ST235, with twenty-seven CPPA isolates testing positive. Of these isolates, approximately 45% (12/27) of ST235 isolates tested significantly positive for the bla_{IMP} gene ($p<0.05$), while four isolates were positive for bla_{GES}, and two isolates were positive for bla_{VIM}. Additionally, co-positivity was observed between bla_{GES} and bla_{VIM}, as well as between bla_{GES}, bla_{VIM}, bla_{NDM}, and bla_{IMP}, each in one isolate. Around 55% (12/22) of bla_{VIM}-positive CPPA isolates belonged significantly to ST357 ($p<0.05$). Similarly, among ST964 isolates, approximately 63% (10/16) tested significantly positive for multiple genes, including bla_{NDM} and bla_{IMP} ($p<0.05$). In addition, six isolates of ST863 were exclusively positive for bla_{VIM}, as were all three isolates of ST1631, which were solely positive for bla_{IMP}. Only ST823, ST621, ST1631, and ST3585 were associated with Carbapenemase genes, and none of these STs had been isolated in Non-Carbapenemase-producing *P. aeruginosa.* Furthermore, this study demonstrates that a sequence typing (ST) group was provided only in the Non-Carbapenemase-producing *P. aeruginosa* strain. ST244 was the most prevalent, followed by ST708 and ST640, with 11 isolates ($p<0.05$) and 5 and 4, respectively. Moreover, five isolates that provided inconclusive ST were only found in the non-carbapenemase-producing group, while one from five isolates that did not completely identify allelic number provided bla_{GES-5}.

Table 12 Distribution of Sequence Types (STs) in CPPA compared with Carbapenemase genes.(N=83)

STs	Number of isolates												
	CP [a]	NDM	IMP	VIM	GES	SIM	GES+VIM	IMP+VIM	NDM+IMP	NDM+VIM	GES+VIM+ IMP+NDM	Non-CP [b]	Total
ST235	23	3	12	2	4	0	1	0	0	0	1	4	27
ST357	15	1	1	12	0	0	0	1	0	0	0	4	19
ST964	15	0	4	0	0	0	0	1	10	0	0	1	16
ST3390	6	1	4	0	0	1	0	0	0	0	0	1	7
ST823	6	0	0	6	0	0	0	0	0	0	0	0	6
ST654	5	3	0	1	0	0	0	0	0	1	0	1	6
ST233	4	2	0	0	1	0	0	0	0	1	0	3	7
ST621	3	1	1	1	0	0	0	0	0	0	0	0	3
ST1631	3	0	3	0	0	0	0	0	0	0	0	0	3
ST708	1	0	1	0	0	0	0	0	0	0	0	5	6
ST277	1	0	1	0	0	0	0	0	0	0	0	1	2
ST3585	1	1	0	0	0	0	0	0	0	0	0	0	1

This study investigated the clonal complex of ST in carbapenem-resistant *P. aeruginosa* (CRPA). All 53 STs were ordered into four groups of single locus variants (SLVs) and double locus variants (DLVs) by BRUST analysis. ST683 and ST970 were classified in Group 1 by DLV, the same as ST3512 and ST202, which were classified in Group 2. Meanwhile, ST654, ST964, and ST244, ST1701 were ordered in group 3 and group 4, respectively, by SLV. In another way, up to 45 STs were unclassified in the clonal complex group.

Table 13 Distribution of MIC50 and MIC90 among predominant STs in Carbapenem-Resistant *P. aeruginosa* (CRPA)

Agents	ST235 (N=27)			ST357 (N=19)			ST964 (N=16)		
	Range of MIC	MIC50	MIC90	Range of MIC	MIC50	MIC90	Range of MIC	MIC50	MIC90
Amikacin	<=8 to >32	<=8[a]	>32	<=8 to >32	<=8[a]	16[a]	<=8 to >32	>32	>32
Gentamicin	<=2 to >8	>8	>8	<=2 to >8	>8	>8	<=2 to >8	>8	>8
Netilmicin	<=8 to >16	>16	>16	<=8 to >16	<=8[a]	16	<=8 to >16	>16	>16
Piperacillin/ Tazobactam	16 to >64	32	>64	16 to >64	>64	>64	<=8 to >64	>64	>64
Ceftazidime	8 to >32	>32	>32	8 to >32	>32	>32	32 to >32	>32	>32
Ceftolozane/Tazobactam	1 to >256	>256	>256	8 to >256	>256	>256	2 to >256	>256	>256
Cefiderocol	<=0.12 to 16	4[a]	8	<=0.12 to 32	8	16	<=0.12 to 16	4[a]	8
Ceftazidime/Avibactam	1 to >64	>64	>64	1 to >64	>64	>64	8 to >64	>64	>64
Gatifloxacin	0.5 to >16	16	>16	0.25 to >16	>16	>16	4 to >16	>16	>16
Imipenem/ Relebactam	0.25 to >64	32	>64	0.5 to >64	>64	>64	2 to >64	>64	>64
Cefepime	4 to >32	>32	>32	8 to >32	>32	>32	32 to >32	>32	>32
Doripenem	1 to >16	>16	>16	<=0.5 to >16	>16	>16	16 to >16	>16	>16
Imipenem	2 to >16	>16	>16	8 to >16	>16	>16	16 to >16	>16	>16
Meropenem	8 to >16	>16	>16	<=0.5 to >16	>16	>16	>16 to >16	>16	>16
Ciprofloxacin	0.5 to >2	>2	>2	1 to >2	>2	>2	1 to >2	>2	>2
Levofloxacin	1 to >8	>8	>8	2 to >8	>8	>8	4 to >8	>8	>8
Colistin	<=1 to >8	2	2	<=1 to >8	2	8	<=1 to 4	2	2

Table 13 presents the sequence typing data for the significant Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) strains and the MIC_{50} and MIC_{90} values for various antimicrobial agents. ST235 is the predominant sequence type, with 27 isolates displaying MIC_{50} values of ≤8 mg/L for Amikacin and four mg/L for cefiderocol, interpreted as susceptible according to CLSI guidelines. ST357, considered a sub-predominant sequence type in this study, demonstrates susceptibility with MIC_{50} values of ≤8 mg/L for both Amikacin and netilmicin. Of the sixteen ST964 isolates, susceptibility is observed exclusively with cefiderocol, with an MIC_{50} value of 4 mg/L. In contrast, ST244 predominates in the non-carbapenemase-

producing group. These findings indicate that a more significant number of agents fall within the susceptible category for MIC_{50} values, including amikacin, gentamicin, netilmicin, ceftolozane/ tazobactam, cefiderocol, ceftazidime/avibactam, and imipenem/relebactam, with MIC_{50} values of ≤8 mg/L, ≤2 mg/L, ≤8 mg/L, ≤0.12 mg/L, 8 mg/L, and 1 mg/L, respectively. It is worth noting that all predominant sequence types exhibit MIC_{50} values categorised as intermediate, with MIC values lower than 4 mg/L.

Table 14 Comparison of sequence type (ST) with Carbapenemase gene and antimicrobial agent susceptibility by MIC_{50}.

STs	Carbapenemase genes	Susceptible to agents [a]:
ST235	*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{GES}$	AN, FDC, COL[c]
ST357	*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{GES}$	AN, NET, COL[c]
ST964	*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$	FDC, COL[c]
ST244	-[b]	AN, GM, C/T, FDC, CZA, IMR, COL[c]

[a] AN; Amikacin, FDC; Cefiderocol, NET; Netilmicin, COL; Colistin, GM; Gentamicin, C/T; Ceftolozane/Tazobactam, CZA; Ceftazidime/Avibactam, IMR; Imipenem/Relebactam.[b] All ST244 were exclusively found in the non-carbapenemase-producing group.[c] Colisin: including MIC_{50} lower than 4 mg/L.

The data from MLST, PCR, and patterns of antimicrobial susceptibility testing have been summarised in Table 14. This study compared the four predominant sequence types with the expression of carbapenemase genes and their susceptibility profiles as interpreted by CLSI guidelines. ST235 is associated with *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{GES}$ carbapenemase genes while demonstrating susceptibility to Amikacin, cefiderocol, and colistin. ST357 was found in isolates positive for *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{GES}$ carbapenemase genes and remained susceptible to amikacin, netilmicin, and colistin. ST964 was positive for *bla*$_{NDM}$, *bla*$_{IMP}$, and *bla*$_{VIM}$ carbapenemase genes and exhibited susceptibility to cefiderocol and colistin. Remarkably, ST244, a significant sequence type in non-carbapenemase-producing

Pseudomonas aeruginosa, remained susceptible to several drugs, including amikacin, gentamicin, ceftolozane/tazobactam (C/T), cefiderocol, ceftazidime/avibactam (CZA), imipenem/relebactam (IMR), and colistin.

CHAPTER V

DISCUSSION

Pseudomonas aeruginosa is widely distributed as a nosocomial pathogen and a major cause of infections worldwide (1). Infections caused by this organism present therapeutic challenges due to its intrinsic resistance and acquired resistance mechanisms. This species naturally expresses various systems, including AmpC β-lactamase, efflux pump, and outer membrane permeability, which contribute to decreased susceptibility rate to various antibiotic groups, including aminoglycosides and fluoroquinolones (1,7). In addition, *P. aeruginosa* can acquire and produce a variety of β-lactamase enzymes like AmpC cephalosporinases, extended-spectrum β-lactamases (ESBLs), and Ambler classes A, B, and D carbapenemases (4,12,13). Of these, metallo-β-lactamases (MBLs; class B) provide an essential function in carbapenem resistance and have been widely published worldwide (3,15).

This study showed that *bla*$_{VIM}$ (26.51%), the most prevalent carbapenemase gene, followed by *bla*$_{IMP\ (IMP\text{-}3/15/19\ group)}$ (22.89%) and *bla*$_{NDM}$ (14.46%). This distribution of genes is consistent with previous studies in Thailand, which have reported that metallo-β-lactamases (MBLs), including *bla*$_{IMP}$ and *bla*$_{VIM}$, are predominant among carbapenem resistance mechanisms in *P. aeruginosa* (24). For example, Khuntayaporn et al. reported *bla*$_{IMP}$ and *bla*$_{VIM}$ were the most detected genes in MBL-producing isolates (24). The high prevalence of *bla*$_{NDM}$ observed in this study further supports recent findings indicating the increasing dissemination of the *bla*$_{NDM}$ carbapenemase gene in Southeast Asia (3). Although traditionally associated with Enterobacterales, *bla*$_{NDM}$ has been increasingly reported in *P. aeruginosa*, contributing to the expanding diversity of resistance mechanisms (3). Notably, the multiple carbapenemase gene isolates, such as *bla*$_{NDM}$ co-with *bla*$_{IMP}$ (12.05%), were observed. This phenomenon has been reported previously and is associated with horizontal dissemination of genes through mobile genetic elements such as plasmids and integrons. (3,15). In addition, a few of the detected genes, such as *bla*$_{GES}$ and *bla*$_{SIM}$, were identified. This finding is relevant to the limitations of the modified carbapenem inactivation method (mCIM), which has been shown to reduce sensitivity for certain

enzyme types (21). Overall, epidemiological trends in MBLs, especially *bla*$_{IMP}$ and *bla*$_{VIM,}$ remain predominant, while *bla*$_{NDM}$ is emerging in CPPA. These results highlight the importance of combining phenotypic and molecular methods for accurate detection and effective surveillance of carbapenemase-producing *P. aeruginosa* (1).

Furthermore, this study examined the distribution of carbapenemase genes among both mCIM-negative and mCIM-positive carbapenemase-producing *P. aeruginosa* (CPPA). Among mCIM-negative isolates, carbapenemase genes were still detected, with *bla*$_{VIM}$, *bla*$_{GES}$, and *bla*$_{IMP}$ accounting for approximately 40%, 33%, and 20% of cases, respectively. The presence of carbapenemase genes in mCIM-negative isolates highlights an important limitation of phenotypic detection methods, which may fail to identify certain enzyme types or low-level expression of carbapenemases (21).

In contrast, mCIM-positive isolates were predominantly associated with *bla*$_{IMP}$ (approximately 35%), followed by *bla*$_{VIM}$ (22%) and *bla*$_{NDM}$, including its combinations (approximately 35%). This result is correlated with previous reports that metallo-β-lactamases (MBLs), particularly *bla*$_{IMP}$ and *bla*$_{VIM}$, are the major contributors to carbapenem resistance in *Pseudomonas aeruginosa* (3,24), while *bla*$_{NDM}$ has been increasingly reported in recent years (3). The co-existence of multiple carbapenemase genes was observed in both mCIM-negative and mCIM-positive groups. For example, isolates harbouring combinations such as *bla*$_{GES}$ with *bla*$_{VIM}$, or multiple genes (*bla*$_{GES}$, *bla*$_{NDM}$, *bla*$_{VIM}$, and *bla*$_{IMP}$), demonstrated positive mCIM results. This observation supports previous findings that the presence of multiple resistance genes may enhance detectable carbapenemase activity and contribute to phenotypic variability (3,15). Subtype analysis revealed variability in mCIM performance depending on specific gene variants. The *bla*$_{IMP}$ gene was the most prevalent overall, with the majority of isolates detected in the mCIM-positive group. However, certain variants, such as *bla*$_{IMP-62}$, were associated with negative mCIM results despite the presence of carbapenemase genes. Similarly, *bla*$_{VIM-2}$ and *bla*$_{VIM-63}$ were identified in both mCIM-negative and mCIM-positive groups, whereas *bla*$_{VIM-5}$ and *bla*$_{VIM-1}$ were detected only in mCIM-positive isolates. These findings suggest that mCIM performance may vary depending on enzyme subtype and expression level. Notably, the *bla*$_{GES}$ group, particularly *bla*$_{GES-5}$, was predominantly associated with

mCIM-negative results. Only one isolate carrying *bla*$_{\text{GES-5}}$ yielded a positive mCIM result when co-existing with *bla*$_{\text{VIM-2}}$. This observation is consistent with previous reports indicating that *bla*$_{\text{GES}}$-type carbapenemases may exhibit weak carbapenem-hydrolysing activity and are often underdetected by standard phenotypic methods (21). Overall, these findings suggest that while mCIM is a reliable method for detecting common carbapenemases, particularly MBLs such as *bla*$_{\text{IMP}}$ and *bla*$_{\text{VIM,}}$ caution is required when interpreting negative results, especially in the presence of specific variants such as *bla*$_{\text{GES}}$ or certain *bla*$_{\text{IMP}}$ and *bla*$_{\text{VIM}}$ subtypes. Therefore, combining phenotypic methods with molecular techniques is essential for accurate detection and effective surveillance of carbapenemase-producing *P. aeruginosa*, in line with current clinical laboratory recommendations (20).

The modified carbapenem inactivation method (mCIM) is widely recognised as a recommended phenotypic approach for detecting carbapenemase producing strain and by the Clinical and Laboratory Standards Institute (CLSI) for Enterobacterales and *Pseudomonas aeruginosa* (20). Previous studies have demonstrated that mCIM shows high accuracy for carbapenemase-producing Enterobacterales (CPE), particularly in regions such as Southeast Asia where metallo-β-lactamases (MBLs) are prevalent (29). However, its performance in *P. aeruginosa* remains less well characterised, and the assay cannot differentiate between serine carbapenemases and MBLs (21). In the present study, mCIM performance was assessed using 164 carbapenem-resistant *Pseudomonas aeruginosa* infection isolates. Of the 83 carbapenemase-producing isolates, 68 were accurately detected by mCIM, corresponding to a sensitivity of about 82%. In contrast, all 81 non-carbapenemase-producing isolates yielded negative results, demonstrating complete specificity. These findings are consistent with previous reports indicating that mCIM typically provides excellent specificity, although its sensitivity may differ according to the carbapenemase type and the bacterial species involved. (21). The reduced sensitivity observed in this study was associated with specific carbapenemase genes, including *bla*$_{\text{VIM-2}}$, *bla*$_{\text{VIM-63}}$, *bla*$_{\text{IMP-62}}$, and *bla*$_{\text{GES-5}}$, which together accounted for more than 15% of CPPA isolates. This suggests that the diagnostic performance of mCIM is influenced by the local distribution of carbapenemase genes. Similarly, these findings have been reported in earlier studies, where certain enzyme variants, particularly *bla*$_{\text{GES}}$ carbapenemases,

were associated with false-negative mCIM results due to their relatively weak carbapenem-hydrolysing activity (21). Gill et al. reported that only approximately 50% of GES-producing isolates yielded positive results with standard mCIM, particularly for variants such as *bla*$_{GES-1}$, *bla*$_{GES-19}$, and *bla*$_{GES-20}$. The authors suggested that the low catalytic efficiency of GES enzymes may limit carbapenem inactivation, leading to false-negative results. They further demonstrated that increasing the bacterial inoculum or prolonging the incubation period could improve detection sensitivity. Similarly, Lisboa et al. reported that mCIM failed to detect several *P. aeruginosa* isolates carrying *bla*$_{GES-5}$. In agreement with these findings, all *bla*$_{GES-5}$ isolates in the present study were negative by mCIM, further supporting the limitation of this method for detecting certain carbapenemase types. In addition, the presence of multiple carbapenemase genes within a single isolate may influence phenotypic detection. Co-expression of enzymes can either enhance or mask detectable activity depending on enzyme kinetics and expression levels, contributing to variability in mCIM results (19). The increasing prevalence of genes such as *bla*$_{NDM}$ in *P. aeruginosa* further complicates detection and reflects the evolving epidemiology of antimicrobial resistance (3). An alternative modification of mCIM using a doripenem disk was evaluated in this study; however, no difference in performance was observed compared with the standard meropenem-based method. This finding suggests that simply changing the carbapenem substrate may not overcome the intrinsic limitations related to enzyme activity or expression.

This study highlights three major findings. Firstly, the mechanisms of carbapenem resistance in the organism were distributed between carbapenemase-producing (CP) and non-carbapenemase-producing (non-CP) groups at comparable proportions. Second, the majority of carbapenemase enzymes identified were metallo-β-lactamases (MBLs), primarily mediated by *bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{NDM}$, consistent with previous reports in Thailand and Southeast Asia (3,24). Third, although mCIM demonstrated excellent specificity, its sensitivity was suboptimal, largely due to its limited ability to detect certain carbapenemase variants, particularly *bla*$_{GES}$ and specific subtypes of *bla*$_{VIM}$ and *bla*$_{IMP}$ (21). These findings underscore that the diagnostic performance of mCIM is highly dependent on the regional distribution of carbapenemase genes (3). Therefore, combining phenotypic methods with molecular

techniques is essential for accurate detection and effective surveillance of carbapenemase-producing *P. aeruginosa*, in line with current clinical laboratory recommendations (20). Although this study was conducted using isolates primarily from Bangkok, Thailand, it provides valuable insight into local resistance patterns and associated diagnostic challenges. Further multicenter studies are warranted to better elucidate the nationwide epidemiology of carbapenem-resistant *P. aeruginosa* and to optimise detection strategies for clinical practice (37,38).

This study investigated the phenotypic resistance patterns of carbapenem-resistant Pseudomonas aeruginosa (CRPA), with particular emphasis on MIC_{50} and MIC_{90} values. The results demonstrated a wide range of susceptibility profiles among tested antimicrobial agents. Notably, most aminoglycosides exhibited MIC_{50} values within the susceptible range, except for gentamicin, which showed both MIC_{50} and MIC_{90} values within the resistant range. Aminoglycosides, including tobramycin, gentamicin, and amikacin, remain important components of antipseudomonal treatment, particularly in infections such as bacteremia, endocarditis, and pulmonary infections in bronchiectasis or cystic fibrosis patients(1).

Ceftolozane–tazobactam (C/T) has been previously reported as an effective treatment option for infections caused by multidrug-resistant (MDR) *P. aeruginosa*, particularly in pneumonia (30). However, its efficacy may be compromised by resistance mechanisms such as AmpC overexpression and other β-lactamase-mediated pathways. In addition, reduced clinical success has been observed in critically ill patients, particularly those with sepsis or undergoing continuous renal replacement therapy (CRRT) (30). In addition, reduced clinical success has been observed in patients, particularly with bacteremia or continuous renal replacement therapy (CRRT) (30). In contrast to previous reports, the present study demonstrated a markedly high MIC_{50} value (>256 mg/L) for C/T, indicating reduced susceptibility for CRPA isolates.

Ceftazidime–avibactam (CZA) has shown activity against Ambler class A, C, and some class D enzymes (12,31). Although CZA has demonstrated clinical efficacy against *P. aeruginosa*, the resistance rate has been increasingly. Previous studies have shown that susceptibility rates of CZA against *P. aeruginosa* are generally lower than those observed in Enterobacterales, with resistance rates ranging from 2.9% to 18% (31). Mechanisms contributing to CZA resistance include amino acid substitutions in

β-lactamases, alterations in outer membrane proteins, and overexpression of efflux pumps (3,31). Consistent with these findings, the present study demonstrated high MIC_{50} values for CZA, suggesting limited effectiveness in CRPA isolates. When comparing carbapenemase-producing (CP) with non-carbapenemase-producing (non-CP) isolates, low susceptibility rates were present for gentamicin, C/T, and CZA in both groups, indicating that resistance is not only dependent on carbapenemase production but may also involve other mechanisms such as efflux pumps and porin loss expression (3).

Cefiderocol, categorised as a novel siderophore cephalosporin agent, has emerged as a promising therapeutic option against organisms with multidrug-resistant (MDR) Gram-negative pathogens (32,33). Published clinical studies have demonstrated the efficacy of this treatment against multidrug-resistant (MDR) and extensively drug-resistant (XDR) pathogens responsible for hospital-acquired pneumonia, bloodstream infections, and urinary tract infections. (32). Resistance to cefiderocol remains relatively uncommon, although multiple mechanisms have been described (33). Importantly, isolates resistant to C/T, CZA, and imipenem–relebactam (IMR) have often retained susceptibility to cefiderocol (32). This observation is consistent with the findings of the present study, where cefiderocol exhibited low MIC values (1 mg/L in non-carbapenemase-producing isolates and 4 mg/L in carbapenemase-producing isolates), indicating susceptibility according to CLSI criteria (20). These results highlight cefiderocol as a potential treatment option for CRPA infections, particularly in cases with limited therapeutic alternatives. This study evaluated the rates of non-susceptibility to various antimicrobial agents among carbapenemase-producing *Pseudomonas aeruginosa* (CPPA) isolates according to their underlying carbapenemase genes. Colistin demonstrated consistently low non-susceptibility rates, ranging from approximately 0% to 28% across all carbapenemase types. These results support the established use of colistin as a critical therapeutic option for infections caused by multidrug-resistant Gram-negative bacteria. Nevertheless, earlier studies have documented a growing prevalence of colistin resistance among MDR and XDR *Pseudomonas aeruginosa* infection strains, emphasising the importance of prudent antimicrobial stewardship and ongoing resistance monitoring. (35). Amikacin, an aminoglycoside antibiotic, showed strong

activity against CPPA isolates, particularly those harbouring *bla*$_{IMP}$ and *bla*$_{VIM}$, which were the predominant carbapenemase genes identified in this study. The low non-susceptibility rates observed in these groups are consistent with previous reports demonstrating that aminoglycosides may retain activity against certain metallo-β-lactamase (MBL)-producing strains (3,7). Nevertheless, resistance mechanisms such as aminoglycoside-modifying enzymes and efflux pump overexpression may still compromise their effectiveness (3). Cefiderocol, a novel siderophore cephalosporin, exhibited potent activity against most CPPA isolates, including those producing *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{GES}$ carbapenemases, as reflected by the low non-susceptibility rates investigated in this study. These findings are in agreement with earlier studies demonstrating that cefiderocol maintains stability against β-lactamase enzymes, including carbapenemases, and retains potent activity against multidrug-resistant organisms (32). However, a relatively higher non-susceptibility rate was observed among CPPA isolates harbouring *bla*$_{NDM}$. This finding may be explained by the fact that *bla*$_{NDM}$-producing strains often possess multiple resistance mechanisms, including co-expression of other β-lactamases, permeability defects, and efflux systems, which may reduce the activity of cefiderocol (3).

This study investigated the sequence types (STs) of carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) using multilocus sequence typing (MLST) in 164 non-duplicate isolates. The CRPA population demonstrated substantial genetic diversity, consistent with the known non-clonal population structure of *P. aeruginosa* (3). Among the identified sequence types, ST235 was the predominant clone, accounting for approximately 16% of all isolates. These results correspond with earlier studies describing ST235 as a globally distributed high-risk clone of *Pseudomonas aeruginosa* infection that is frequently linked to resistance against fluoroquinolones, aminoglycosides, β-lactams, and carbapenems. (36). A prior study conducted in Thailand similarly reported ST235 as the dominant sequence type, followed by ST964 and ST111, with ST235 present in both carbapenemase-producing and non-producing isolates (24,37). In this study, ST235 was identified in both isolate groups but was predominantly observed among carbapenemase-producing *Pseudomonas aeruginosa* infection isolates, accounting for 25 of 27 strains, further supporting its significance as a major high-risk clone in this setting. Other sequence types, including ST357, ST964,

ST654, ST233, ST260, ST277, and ST708, were identified in both CPPA and non-CPPA groups, indicating that carbapenem resistance is not restricted to a single lineage but is likely driven by horizontal gene transfer and independent acquisition of resistance determinants (3). In contrast, certain STs, such as ST823, ST3390, ST621, ST1631, and ST3585, were exclusively found in CPPA isolates, suggesting possible clonal expansion associated with carbapenemase gene carriage. Interestingly, ST244 was exclusively identified in the non-carbapenemase-producing group in this study. This observation contrasts with previous reports from China and Europe, where ST244 has been associated with carbapenemase genes such as *bla*$_{IMP}$ and *bla*$_{VIM}$ (38). These discrepancies highlight the geographic variability in the distribution of high-risk clones and emphasise the importance of local epidemiological surveillance. About carbapenemase gene distribution, ST235 was most frequently associated with *bla*$_{IMP}$, followed by *bla*$_{GES}$, whereas ST357 was predominantly associated with *bla*$_{VIM}$. Notably, all ST823 isolates in this study carried *bla*$_{VIM}$, which is consistent with previous findings reporting a strong association between ST823 and *bla*$_{VIM}$-type metallo-β-lactamases (3). In addition, co-occurrence of *bla*$_{NDM}$ and *bla*$_{IMP}$ was exclusively observed in ST964 isolates, suggesting a potential role of this lineage in the accumulation of multiple resistance determinants. Analysis of antimicrobial susceptibility profiles among predominant STs revealed distinct patterns. ST244, the dominant sequence type among non-carbapenemase-producing isolates, demonstrated MIC_{50} values within the susceptible range for several agents, including amikacin, imipenem–relebactam, and colistin (MIC_{50} < 4 mg/L), according to CLSI criteria (20). In contrast, predominant CPPA-associated clones such as ST235 and ST357 retained susceptibility to selected agents, including amikacin, colistin, and cefiderocol (for ST235), and netilmicin (for ST357), despite harboring multiple carbapenemase genes. Similarly, ST964 isolates showed susceptibility to cefiderocol and colistin. These findings suggest that antimicrobial susceptibility patterns may vary according to sequence type and associated resistance mechanisms. Therefore, integrating MLST data with carbapenemase gene profiling and phenotypic susceptibility testing may provide valuable insights for guiding targeted antimicrobial therapy and improving clinical management of CRPA infections.

In summary, this study provides comprehensive insights into the antimicrobial resistance landscape of carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) by integrating phenotypic susceptibility profiles, carbapenemase gene characterisation, and molecular epidemiology through MLST analysis. The findings demonstrate that metallo-β-lactamases, particularly *bla*$_{VIM}$ and *bla*$_{IMP}$, remain the predominant resistance mechanisms in this setting, while the emergence of *bla*$_{NDM}$ and the co-existence of multiple resistance genes highlight the increasing genetic complexity of CRPA (3,4,24). Although the modified carbapenem inactivation method (mCIM) showed high specificity, its limited sensitivity for certain enzymes, especially *bla*$_{GES}$-type carbapenemases, underscores the importance of combining phenotypic and molecular methods for accurate detection (20,21). From a therapeutic perspective, variability in antimicrobial susceptibility was observed across different resistance mechanisms and sequence types. While agents such as amikacin, colistin, and cefiderocol retained activity against selected isolates, decreased susceptibility to β-lactam/β-lactamase inhibitor combination agents has raised concerns regarding treatment effectiveness. The MLST analysis further revealed a diverse population structure, with high-risk such as ST235 playing a dominant role in the dissemination of resistance, alongside the presence of emerging and region-specific sequence types.

CHAPTER VI

CONCLUSION

This study demonstrated the molecular epidemiology, carbapenemase gene distribution, antimicrobial susceptibility patterns, and efficacy of the modified inactivated method (mCIM) for detecting carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) isolates. Of the 164 CRPA isolates, carbapenemase genes were identified in approximately 50% from total, with bla_{IMP} being the predominant gene, followed by bla_{VIM} and bla_{NDM}. The findings also revealed substantial genetic diversity, with 53 sequence types (STs) identified; however, only a limited number of STs were associated with carbapenemase production. ST235, ST357, and ST964 were the major carbapenemase-producing lineages, whereas ST244 was the predominant non-carbapenemase-producing CRPA clone. Distinct associations between specific STs and carbapenemase genes were observed, suggesting clonal dissemination of resistant strains in Thailand.

The modified Carbapenem Inactivation Method (mCIM) showed incomplete sensitivity for detecting carbapenemase-producing isolates, particularly among strains harbouring bla_{VIM}, bla_{IMP-62}, and bla_{GES-5}. These findings indicate that reliance on phenotypic methods alone may lead to underdetection of carbapenemase-producing *P. aeruginosa*, emphasising the importance of molecular approaches for accurate identification.

Antimicrobial susceptibility testing demonstrated extensive multidrug resistance among CRPA isolates. Cefepime-zidebactam exhibited the most promising in vitro activity, with both MIC_{50} and MIC_{90} within the susceptible range, while cefiderocol retained partial activity. Aminoglycosides, particularly amikacin and netilmicin, also showed relatively favourable activity compared with other agents. In contrast, most conventional antipseudomonal antibiotics were largely ineffective, highlighting the limited therapeutic options currently available for CRPA infections. Notably, ST357 isolates displayed the highest resistance levels among the predominant carbapenemase-producing clones.

Overall, this study provides important insights into the epidemiology, resistance mechanisms, and antimicrobial susceptibility profiles of CRPA in Thailand. The findings underscore the growing threat posed by high-risk resistant clones and emphasise the necessity for continuous molecular surveillance, accurate carbapenemase detection, and the development of effective antimicrobial strategies to control the spread and clinical impact of CRPA infections.

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APPENDIX