

Research Article

Antibiotic Profiling and Detection of *bla*_{IMP-1} and *bla*_{VIM-2} in Carbapenem-Resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from Selected Tertiary Hospitals in Metro Manila, Philippines

Sherill Tesalona^{1*}, Evelina Lagamayo², Edmalin Tuban¹, Mary Genevieve Ello¹, Rika Hoshina¹, Maria Evangeline Millado¹, Earl Justin Sale¹, Ansherina Joi Sevilla¹, Fayye Ann Eloise Solquio¹, Mary Angeli Tienzo¹, Randvyr Timtiman¹, and Maria Ruth Pineda-Cortel^{1,3}

¹Department of Medical Technology, University of Santo Tomas, Philippines

²Department of Laboratory Medicine, University of Santo Tomas, Philippines

³Research Center for the Natural and Applied Sciences, University of Santo Tomas, Philippines

*Corresponding author

Sherill Tesalona, Department of Medical Technology, Faculty of Pharmacy, University of Santo Tomas, 1015 Manila, Philippines; Tel: 639178283595; E-mail: sdtosalona_ustgs11@yahoo.com/sherilldtesalona@gmail.com

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- PCR
- Antibiotic susceptibility
- *bla*_{IMP-1}, *bla*_{VIM-2}
- *Acinetobacter baumannii*
- *Pseudomonas aeruginosa*

Abstract

Introduction: *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are common opportunistic bacteria causing nosocomial infections among hospitalized patients. Aside from being opportunistic, these bacteria, nowadays, are exhibiting resistance to some antibiotics, making antibiotic therapy difficult and challenging. With this, we aimed to determine the presence of some resistance-causing genes such as *bla*_{IMP-1} and *bla*_{VIM-2} from carbapenem-resistant *A. baumannii* (CRAB) and carbapenem-resistant *P. aeruginosa* (CRPA).

Methodology: Purposive sampling was used wherein all CRAB and CRPA isolates were collected from two selected tertiary hospitals in Metro Manila, Philippines. Phenotypic identification and antibiotic susceptibility testing of the isolates were done using VITEK® 2 Compact. Carbapenem resistance was retested using Kirby-Bauer disk diffusion method. For the genotypic identification of the presence of *bla*_{IMP-1} and *bla*_{VIM-2}, the deoxyribonucleic acid (DNA) of the samples was isolated and purified. It was then amplified through conventional polymerase chain reaction (PCR). Amplicons produced were run through gel electrophoresis for the visualization of bands indicating the presence of *bla*_{IMP-1} at 740 bp and *bla*_{VIM-2} at 865 bp.

Results: We detected the genes *bla*_{IMP-1} and *bla*_{VIM-2} in four (58%) and one (14%) CRAB isolates, respectively. One (14%) isolate has both *bla*_{IMP-1} and *bla*_{VIM-2}. While in CRPA isolates, only *bla*_{IMP-1} was detected in two (67%) isolates. In terms of the antibiotic profiling of the isolates with *bla*_{IMP-1} and *bla*_{VIM-2} we found that all are resistant against ceftazidime and ciprofloxacin (100%); seven (87.5%) against cefepime, gentamicin, and piperacillin/tazobactam; and one (12.5%) against amikacin. Furthermore, the antibiotic profile of the isolates with no *bla*_{IMP-1} and *bla*_{VIM-2} are the following: all are resistant against cefepime, ceftazidime, ciprofloxacin, gentamicin, and piperacillin/tazobactam (100%). On the other hand, both *bla*_{IMP-1} and *bla*_{VIM-2} negative isolates are sensitive to amikacin. All isolates (100%) are sensitive against colistin.

Conclusion: We conclude that *bla*_{IMP-1} is present in CRAB and CRPA, and it is more frequent than *bla*_{VIM-2}. This study also concludes the coexistence of multiple metallo-β-lactamase genes in CRAB isolates.

ABBREVIATIONS

CRPA: Carbapenem-Resistant *P. aeruginosa*; CRAB: Carbapenem-Resistant *A. baumannii*; IMP: Imipenem Hydrolyzing Carbapenemases; VIM: Verona-Integron Metallo-β-

lactamase; PCR: Polymerase Chain Reaction

INTRODUCTION

Acinetobacter baumannii and *Pseudomonas aeruginosa* have emerged globally, especially in the hospital setting, as these cause

fatal nosocomial infections, such as hospital-acquired pneumonia, urinary tract infection, bacteremia, and chronic obstructive pulmonary disease [1,2]. These gram-negative bacteria have been reported to exhibit multidrug resistance on carbapenems and third-generation cephalosporins, and are classified as the most critical groups in need for new alternative antibiotics [3,4]. The increasing prevalence of carbapenem-resistance in major nosocomial pathogens, such as *A. baumannii* and *P. aeruginosa*, necessitates intensive measures for diagnosis and treatment as well as correct use of antibiotics.

MATERIALS AND METHODS

Sampling technique

Purposive sampling technique was used in this study.

Specimen collection, transport, and storage

All CRAB and CRPA isolates were from November 2016 to March 2017 from two selected tertiary hospitals in Metro Manila, Philippines. Only 10 samples were gathered. All the collected samples were pre-identified to be resistant to carbapenem through antibiotic susceptibility testing (AST) using VITEK® 2

Compact conducted in the laboratory where the samples were obtained. Patients' names were de-identified from the isolates, and only case numbers were used to protect patients' privacy. The isolates were inoculated into nutrient agar (NA) for transport to the UST Faculty of Medicine and Surgery - Department of Laboratory Medicine, where the specimens were further processed. All biosafety measures recommended by the World Health Organization (WHO) on proper specimen collection, transport, and disposal were followed.

Phenotypic testing

Bacterial identification and Antibiotic susceptibility testing (AST): Pre-identification of all the isolates up to species level and antibiotic susceptibility testing were done using VITEK® 2 Compact. Susceptibility for carbapenem was retested using the Kirby-Bauer disk diffusion method following the CLSI Guidelines 2017 [5].

Genotypic testing

DNA lysate preparation: For the preparation of DNA lysate, a single-step liquid-phase separation method based on the Wizard® Genomic DNA Purification Kit Technical Manual by Promega (2014) was used.

DNA purification using Nanodrop: Measurement of DNA concentration is an important procedure in molecular biology. This ensures that all isolated DNA samples are in its purest form and free of any impurities or protein contaminations. Absorption spectroscopy method was used in this experiment using a spectrophotometer machine. For quantitative measurements of pure nucleic acid, absorbance with a wavelength of A_{260} , A_{280} , and A_{320} were used [6].

Polymerase chain reaction (PCR)

For PCR, a Master Mix using Promega M782A was prepared. This contained Go Taq G2 Green Master Mix that includes DNA polymerase enzyme (Taq: *Thermus aquaticus*). PCR nuclease water as well as forward and reverse primers of *bla*_{IMP-1} and *bla*_{VIM-2} were added to the master mix to delineate a specific region to be amplified.

Controls: A strain of *A. baumannii* with a known *bla*_{IMP-1} confirmed by DNA sequencing and BLAST was used as a positive control. The positive control has query coverage of 95.0% and an 88.0% similarity based on BLAST search of *bla*_{IMP-1} sequence [7].

Primers: The primers used for amplification were: (1) IMP-1 Forward TGAGCAAGTTATCTGTATTC and Reverse TTAGTTGCTTGGTTTTGATG with a size of 740 bp amplicon and (2) VIM-2 Forward AAAGTTATGCCGCACTCACC and Reverse TGCAACTTCATGTTATGCCG with a size of 865 bp amplicon [8].

Amplification of *bla*_{IMP-1} and *bla*_{VIM-2}: The DNA amplification kit used for amplifying nucleic acid contained DNA polymerase, primers, nuclease-free water, and template DNA. The reaction mixture was prepared simultaneously as the DNA working lysate were immersed in an ice bath. The mixture was composed of 225 μ L Go Taq G2 Green Master Mix, 153 μ L PCR nuclease-free water, and 9 μ L of each forward and reverse primer. The template

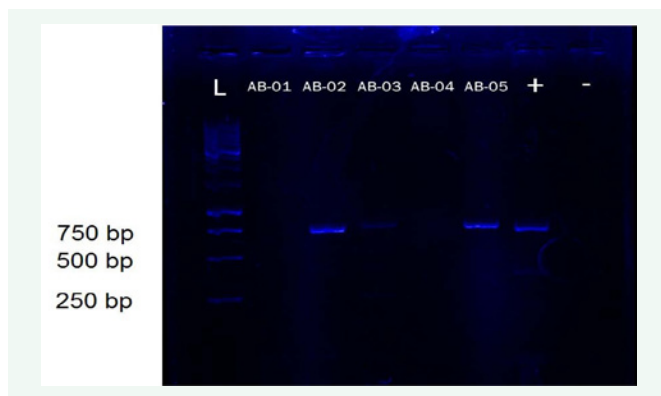


Figure 1a Gel electropherogram for *bla*_{IMP-1} (740 bp) 1st batch. Isolates AB-01, AB-02, AB-03, AB-04, and AB-05 for *bla*_{IMP-1} detection; AB-02, AB-03, and AB-05 are positive; DNA ladder (L); Positive control (+); Negative control (-)

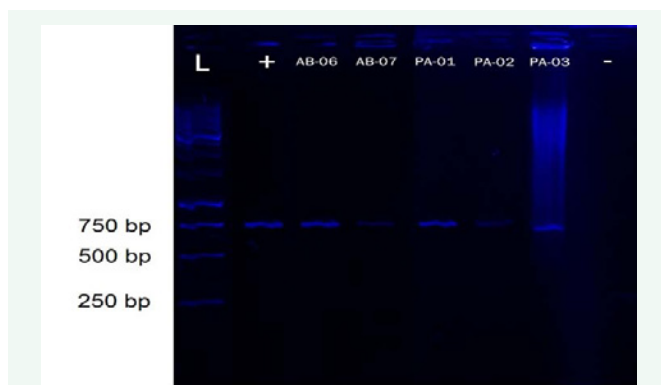


Figure 1b Gel electropherogram for *bla*_{IMP-1} (740 bp) 2nd batch. Isolates AB-06, AB-07, PA-01, PA-02, and PA-03 for *bla*_{IMP-1} detection; AB-06, AB-07, PA-01, and PA-02 are positive; DNA ladder (L); Positive control (+); Negative control (-).

DNA of 3 μ L was added to 22.0 μ L of the reaction mixture. The same process was done to the negative control. Furthermore, the cycling parameters used for *bla*_{IMP-1} and *bla*_{VIM-2} were as follows: the initial incubation step required one cycle at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 7 minutes (Swift MaxPro Thermal Cycler [8]).

Amplified Product Detection (Gel Electrophoresis):

Visualization of PCR products was done by using gel electrophoresis in 1x TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA [pH 8.5]) with 2.0 μ L gel red dye. One percent (1%) agarose gel with 1.0 μ L gel red dye was used to run the amplicon soaked in 1x TAE buffer. An amount of 5.0 μ L of DNA amplicon mixed with 1.0 μ L 6x loading dye was placed to the wells of the prepared 1% agarose gel block located on the electrophoresis tank. For the DNA marker, 5 μ L of 1 kb DNA ladder was used. As a protocol, electrophoresis machine conditions were set to 100V, 500 MA for 60 minutes. The results of the gel electrophoresis were viewed and photographed (C.B.S Scientific EPS-300 IIV and Cleaver Scientific nanoPAC300 mini) [8].

RESULTS AND DISCUSSION

In our study, we were able to gather only 10 isolates from two selected tertiary hospitals in Metro Manila, Philippines. The isolates were collected from nine (9) (90%) hospitalized patients, mostly from intensive care units (ICUs), and only one (1) (10%) from an out-patient; mostly from males (60% males and 40% females); and with ages ranging from 41 to 65 years. Based on the pre-identification conducted by the hospital laboratories where the specimens were obtained, all the isolates are either carbapenem-resistant *A. baumannii* or *P. aeruginosa* using VITEK® 2 Compact.

Table 1A shows that all (8/8) isolates of CRAB and CRPA positive for *bla*_{IMP-1}/*bla*_{VIM-2} have the following antibiotic resistance profile: ceftazidime and ciprofloxacin 8 (100%), cefepime, gentamicin, and piperacillin/tazobactam 7 (87.5%), and amikacin, 1 (12.5%), whereas colistin has 0% resistance. Furthermore, all (100%) CRAB and CRPA isolates negative for *bla*_{IMP-1}/*bla*_{VIM-2} have the following resistance profile: 100% resistance to cefepime, ceftazidime, ciprofloxacin, gentamicin, and piperacillin/tazobactam, whereas it has 0% resistance to amikacin and colistin. Table 1B shows the patterns of antibiotic resistance all 10 strains.

To test for the presence of *bla*_{IMP-1} and *bla*_{VIM-2}, which we hypothesized to be responsible for the resistance profile, we performed DNA isolation and conventional PCR and visualized band formation using gel electrophoresis. Table 2 and Figures 1 and 2 show that four CRAB isolates (58%) were positive for *bla*_{IMP-1} (740 bp) and 1 (14%) for *bla*_{VIM-2} (865 bp). One isolate (14%) had both *bla*_{IMP-1} and *bla*_{VIM-2}, which demonstrated possible coexistence of multiple MBL genes [9]. *Bla*_{VIM-2} was not detected among CRPA isolates. Furthermore, two out of the three CRPA isolates carried the *bla*_{IMP-1} gene. In this research, *bla*_{IMP-1} was more prevalent than *bla*_{VIM-2}.

A study in India showed a similar result with 42% total prevalence of *bla*_{IMP-1} and *bla*_{VIM-2}, wherein *bla*_{IMP-1} was more

prevalent [10]. MBL genes can induce genetic apparatuses to become highly mobile since these are associated with integrons embedded in transposons, hence, making the MBL gene dissemination likely to occur. The higher distribution of *bla*_{IMP-1} in CRPA was also evident in the studies of Tesalona et al., (unpublished) in the Philippines and Azim et al., (2009) [7,11] in India with results of 9 (90%) and 7 (58%), respectively. By contrast, in a study performed in Iran among burn patients, none of the 65 CRAB isolates collected contained *bla*_{IMP}, while eight (12.5%) strains carried *bla*_{VIM}. Six out of eight VIM-producing strains contained both *bla*_{OXA-23} and *bla*_{VIM} genes [18].

CRAB isolates used in the study were from respiratory (42%), exudative (29%), and urine (29%) specimens as shown in Table 3A,B, while CRPA isolates were from respiratory specimen (33%), exudative specimen (33%), and blood (33%). The most frequent specimen where CRAB or CRPA isolates can be isolated cannot be determined in this study because of the limited number of isolates collected. In previous studies, common sources of CRAB and CRPA isolates were those from respiratory and urethral

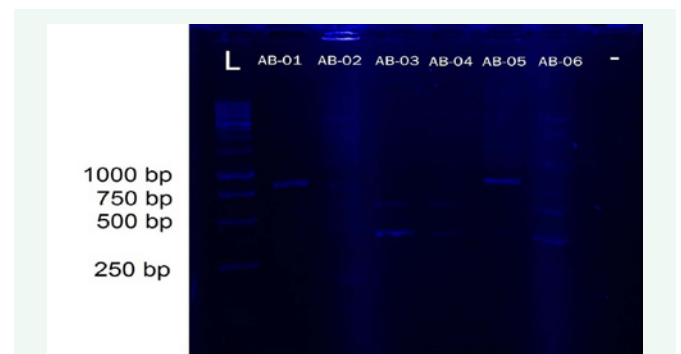


Figure 2a Gel electropherogram for *bla*_{VIM-2} (865 bp) 1st batch. Isolates AB-01, AB-02, AB-03, AB-04, AB-05, and AB-06 for *bla*_{VIM-2} detection; AB-01 and AB-05 are positive; DNA ladder (L); Negative control (-).

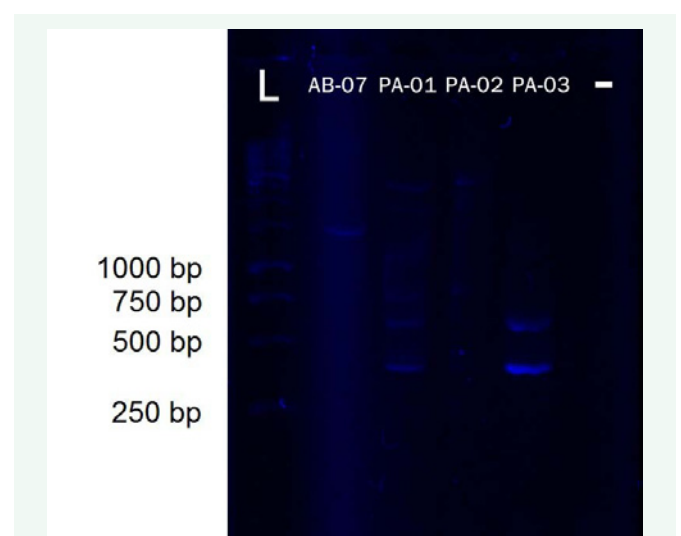


Figure 2b Gel electropherogram for *bla*_{VIM-2} (865 bp) 2nd batch. Isolates AB-07, PA-01, PA-02, and PA-03 for *bla*_{VIM-2} detection and all are negative for *bla*_{VIM-2}, DNA ladder (L); Negative control (-).

catheters. These catheters served as surface for bacterial adhesion that can promote widespread distribution of the bacteria [12]. In the studies of Tesalona et al., (unpublished) and Amudhan et al., (2012), the primary sources of isolates were respiratory secretions and urine specimens [7,9]. On the contrary, wound exudates from burn patients in Iran were the primary source of 39% CRAB and 38% CRPA isolates that were also MBL producers. Burn wounds harbor bacteria to thrive and become sources of infection as larger areas and longer duration of hospitalization are involved [16,17]. Further study involving more isolates may be done to identify the most frequent specimen source of CRAB and CRPA isolates.

The presence of genes causing resistance to antibiotics is an important mechanism to explore in the study of multidrug-resistant bacterial pathogens, especially among bacteria-causing

nosocomial infections. Patients staying in the hospital for prolonged periods are at high risk for such nosocomial infections. Moreover, the risk for such infections is worsened by the spread of multidrug-resistant bacterial strains.

Multidrug-resistance develops due to microbial alterations caused by genetic mutation, modification in efflux mechanism, and changes in cell morphology. The Center for Disease Control (CDC) in Europe recorded a total of 25,000 deaths per year and 2.5 million additional hospital days due to antibiotic resistance, while over 58,000 babies died within a year in India due to resistant bacterial infection transmitted by their mothers.

In this study, we focused on beta-lactam resistance of *A. baumannii* and *P. aeruginosa*. The primary mechanism of beta-lactam resistance employs destruction of beta-lactam ring by

Table 1A: Antibiotic resistance profile of 7 CRAB and 3 CRPA isolates, bla_{IMP-1} - and bla_{VIM-2} -producing and non-producing strains interpreted based on CLSI Guidelines 2017 collected from two selected tertiary hospitals in Metro Manila from November 2016 to March 2017.

Antibiotics	CRAB and CRPA isolates (n=10)		bla_{IMP-1} - and/or bla_{VIM-2} - positive (n=8)		Negative (n=2)		p-value	Conclusion
	n	%	n	%	n	%		
Amikacin	1	10	1	12.5	0	0	1.000	NS
Cefepime	7	70	7	87.5	2	100	1.000	NS
Ceftazidime	8	80	8	100	2	100	1.000	NS
Ciprofloxacin	10	100	8	100	2	100	1.000	NS
Gentamicin	9	90	7	87.5	2	100	1.000	NS
Piperacillin/Tazobactam	9	90	7	87.5	2	100	1.000	NS
Colistin	0	0	0	0	0	0	-	-

Tested at $p=0.05$

CRAB: Carbapenem-resistant *Acinetobacter baumannii*; CRPA: Carbapenem-resistant *Pseudomonas aeruginosa*; IMP: Imipenem hydrolyzing Metallo- β -Lactamase; VIM: Verona-integron encoded Metallo- β -lactamase; NS: No significance

Table 1B: Antibiotic Resistance Profile of all (10) CRAB and CRPA isolates.

Isolates	PCR result		Antibiotics								
	bla_{IMP-1}	bla_{VIM-2}	IMP/MEM	AN	FEP	CAZ	CIP	GN	TZP	COL	
(+) Control bla_{IMP-1}	POS										
(-) Control Nuclease-free water	NEG	NEG									
<i>Acinetobacter baumannii</i>											
1	AB01	NEG	POS	R	NR	R	R	R	R	R	NR
2	AB02	POS	NEG	R	NR	R	R	R	R	R	NR
3	AB03	POS	NEG	R	NR	R	R	R	R	R	NR
4	AB04	NEG	NEG	R	NR	R	R	R	R	R	NR
5	AB05	POS	POS	R	NR	R	R	R	R	R	NR
6	AB06	POS	NEG	R	NR	R	R	R	R	R	NR
7	AB07	POS	NEG	R	NR	R	R	R	R	R	NR
<i>Pseudomonas aeruginosa</i>											
8	PA01	POS	NEG	R	R	NR	R	R	R	NR	NR
9	PA02	POS	NEG	R	NR	R	R	R	NR	R	NR
10	PA03	NEG	NEG	R	NR	R	R	R	R	R	NR

AB: Carbapenem-resistant *Acinetobacter baumannii*; PA: Carbapenem-resistant *Pseudomonas aeruginosa*; IMP: Imipenem; MEM: Meropenem; AN: Amikacin; FEP: Cefepime; CAZ: Ceftazidime; CIP: Ciprofloxacin; GN: Gentamicin; TZP: Piperacillin/Tazobactam; COL: Colistin

Table 2: Distribution of *bla*_{IMP-1} and *bla*_{VIM-2} in 7 CRAB and 3 CRPA isolates collected from two selected tertiary hospitals in Metro Manila from November 2016 to March 2017.

Genotypes	CRAB Isolates (n=7)	CRPA Isolates (n=3)	CRAB %	CRPA %
<i>bla</i> _{IMP-1} only	4	2	58	67
<i>bla</i> _{VIM-2} only	1	0	14	0
both <i>bla</i> _{IMP-1} and <i>bla</i> _{VIM-2}	1	0	14	0
<i>blagene</i> (-)	1	1	14	33
Total	7	3	100	100

CRAB: Carbapenem-resistant *Acinetobacter baumannii*; CRPA: Carbapenem-resistant *Pseudomonas aeruginosa*; IMP: Imipenem hydrolyzing Metallo-β-Lactamase; VIM: Verona-integron encoded Metallo-β- lactamase

Table 3A: Distribution of *bla*_{IMP-1} and *bla*_{VIM-2} in CRAB strains from various specimens collected from selected tertiary hospitals in Metro Manila from November 2016 to March 2017.

Specimens	<i>bla</i> _{IMP-1} / <i>bla</i> _{VIM-2}				Total CRAB	
	Positive		Negative		n	%
	n	%	n	%		
Respiratory specimens	2	29	1	14	3	42
Exudative specimens	2	29	0	0	2	29
Urine	2	29	0	0	2	29
Blood	0	0	0	0	0	0
Total	6	87	1	14	7	100

CRAB: Carbapenem-resistant *Acinetobacter baumannii*; CRPA: Carbapenem-resistant *Pseudomonas aeruginosa*; IMP: Imipenem hydrolyzing Metallo-β-Lactamase; VIM: Verona-integron encoded Metallo-β- lactamase

Table 3B: Distribution of *bla*_{IMP-1} and *bla*_{VIM-2} in CRPA strains from various specimens collected from selected tertiary hospitals in Metro Manila from November 2016 to March 2017.

Specimens	<i>bla</i> _{IMP-1} / <i>bla</i> _{VIM-2}				Total CRPA	
	Positive		Negative		n	%
	n	%	n	%		
Respiratory specimens	0	0	1	33	1	33
Exudative specimens	1	33	0	0	1	33
Urine	0	0	0	0	0	0
Blood	1	33	0	0	1	33
Total	2	66	1	33	3	100

CRAB: Carbapenem-resistant *Acinetobacter baumannii*; CRPA: Carbapenem-resistant *Pseudomonas aeruginosa*; IMP: Imipenem hydrolyzing Metallo-β-Lactamase; VIM: Verona-integron encoded Metallo-β- lactamase

beta-lactamases, hydrolyzing the amide bond of the ring. Genes encoding for these enzymes are termed as *bla* succeeded by their specific name (e.g., *bla*_{IMP}). In addition, these genes can be found as parts of accessory genome or integrons[8]. One of the most resilient beta-lactam enzymes, carbapenemase, has the ability to hydrolyze carbapenems, rendering the bacteria resistant. It has several classes and subclasses depending on the amino acid homology: Classes A, C, and D use serine as their active site; Class B (BI, BII, and BIII) uses zinc as the active site. Subclass BII is further divided into four categories based on their molecular structures: IMP, VIM, GIM, and SPM types [8]. IMP-1 was first discovered in *Serratia marcescens* in Japan in 1991, VIM-1 in Italy in 1997, GIM in Germany in 2002, and SPM-1 in Brazil in 1997[12,13]. These genes are encoded in integrons that specify aminoglycoside 6'-N-acetyltransferases, making easier horizontal transfer among different species of bacteria possible [15]. The gene *bla*_{IMP} "active on imipenem" was first discovered in a *P. aeruginosa* strain GN17203. The isolate possessed an imipenem MIC of 50 µg/ml as

well as resistance to extended-spectrum such as cephalosporins, a ceftazidime MIC of >400 µg/ml. The resistance allele, IMP-1, was found on a transferable conjugative plasmid that could be readily mobilized to other *Pseudomonas* strains [8]. So far, there are 23 different IMP MBLs that have been identified.

Another prevalent MBL family is the "Verona integrin-encoded metallo-beta lactamase" (VIM) that was first isolated in Verona, Italy in the form of VIM-1 and VIM-2. The VIM family is comprised of 14 members, most of which have occurrences in *P. aeruginosa* within multiple-integron cassette structures [8]. The VIM-type MBLs have been widespread in Asia, Southern Europe, and North America. In 2000, Antibiotic Resistance Monitoring and Reference Laboratory had received approximately 80 isolates, mostly *Pseudomonas* species from the United Kingdom, which tested positive for *bla*_{VIM}[13].

In the present study, we were able to detect the presence of both *bla*_{IMP-1} and *bla*_{VIM-2} in our isolates. We only have 10

isolates and yet the two genes that are probable for causing drug resistance, were detected. It is therefore recommended that hospitals should have regular screening strategies to monitor multi-drug resistance especially among bacteria causing nosocomial infections.

CONCLUSION

We conclude that *bla*_{IMP-1} and *bla*_{VIM-2} are present in CRAB and CRPA isolates. *bla*_{IMP-1} in CRAB and CRPA is more prevalent than *bla*_{VIM-2}. The coexistence of multiple genes was also observed. Further studies on the presence of these genes in a larger scale of isolates may be done to be able to identify if the genes are associated with resistance against antibiotics.

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