

Molecular detection and characterisation of Metallo- β -Lactamase (MBL) Genes and integrons of Imipenem –Resistant *Pseudomonas aeruginosa*

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The increase in the prevalence of carbapenem-resistant *Pseudomonas aeruginosa* isolates, particularly caused by the metallo- β -lactamases (MBL) is of great concern in the clinical settings worldwide. Here, we report the detection and identification of MBL producing *P. aeruginosa* in our hospital (University Malaya Medical Center, Malaysia), and the characterization of MBL cassette-containing integrons in 90 imipenem-resistant *P. aeruginosa* isolates. Of the isolates investigated in this study, 32 (94%) MBL-positive strains were identified, harboring either *bla*_{IMP} or *bla*_{VIM} genes. Three MBL phenotypic assays methods (CDT, DDST, and IP/IPI *E*-test) were evaluated by comparison to that of molecular methods. The MBL IP/IPI *E*-test exhibited 100% accuracy in the detection of MBL production. Class I integrons were detected as the most abundant type of integron in the Malaysian isolates. The genes detected from MBL-positive isolates were *bla*_{IMP-7}, *aacC1*; *bla*_{VIM-11}; *aacA7*, *bla*_{VIM-2}, *attI*, and *aacA7*. Our findings show that class I integrons were prevalent in *P. aeruginosa* and they may play an important role in the dissemination of multidrug resistance *P. aeruginosa*. Based on the differences in the fingerprinting patterns, two clusters (A and B) were identified among the MBL-producing isolates. Cluster A comprised 18 isolates (56 %) carrying the *bla*_{VIM} gene, whereas cluster B comprised 14 (44 %) isolates carrying the *bla*_{IMP} gene. The non-MBL isolates were divided into clusters C and D. Cluster C comprised 22 non-MBL isolates harbouring class 1 integrons, whilst cluster D consisted of three isolates carrying class 2 integrons.

1. Introduction

Pseudomonas aeruginosa is a versatile pathogen associated with a broad spectrum of infections in humans. The pathogenicity of these organisms is mainly due to the ability of the organism to produce a variety of toxins and proteases as well as the ability to resist phagocytosis [1]. The bacterial infection is usually associated with high morbidity and mortality in individuals (specify the individuals) due to the difficulties in treatment attributed to the emergence and spread of drug-resistant strains [2]. The most potent antimicrobial agents for the treatment of *P. aeruginosa* infections are carbapenems, which includes imipenem and meropenem, [3]. However, a global increase in the prevalence of carbapenem resistant *P. aeruginosa* is observed and more recently, the emergence of multidrug-resistant (MDR) *P. aeruginosa* has become a serious problem in healthcare settings worldwide [4]. Carbapenem resistance may be mediated by production of certain β -lactamases (MBLs) [5]. The genes encoding for MBL are carried on large transferable plasmids or associated with transposons which allow horizontal transfer of the genes among different bacterial genera and species. Several families of MBLs including IMP, VIM, SPM, GIM, SIM, KHM, AIM and NDM have been reported in *P. aeruginosa*. Among these, IMP and VIM variants have been reported worldwide while SPM, GIM, SIM, KHM, AIM and NDM are mostly restricted to certain geographical regions [5].

Continuous surveillance of *P. aeruginosa* resistance against antimicrobial agents is vital to monitor trends in its susceptibility patterns to permit selection of the best option for empirical or directed therapy. In this study, three phenotypic methods, i.e., combined IPM-EDTA disk test (CDT), double-disk synergy test (DDST), and IP/IPI *E*-test) were evaluated for MBL detection in imipenem-resistant *P. aeruginosa* (IRPA) clinical isolates, in comparison to PCR detection of MBL genes as the gold standard. The different variants of MBL genes present among IRPA clinical isolates from University Malaya Medical Center (UMMC), Kuala Lumpur, Malaysia were also determined. Integrons and their associated gene cassettes were characterized and the genetic relatedness of the isolates was investigated using random amplification of polymorphic DNA (RAPD) analysis.

2. Material and Methods

2.1. Bacterial Isolates

Ninety random non-replicate imipenem-resistant *P. aeruginosa* (IRPA) isolates from various clinical specimens of 90 non-repetative patients (57 females and 33 males) admitted to UMMC were used in this study. The isolate were collected from two periods: i) October 2005 to March 2006 ($n=28$) isolates and ii) October 2007 to March 2008 ($n=62$) isolates. All bacterial isolates were identified as *P. aeruginosa* using API-20NE (BioMerieux, Marcy l'Etoile, France).

2.2. Antimicrobial susceptibility testing and phenotypic tests for detection of MBL genes

Susceptibility to anti-pseudomonal antimicrobials, such as ciprofloxacin, piperacillin, gentamicin, ceftazidime, amikacin, netilmicin, and cefloperazone was performed by disk diffusion method, according to Clinical and Laboratory

Standards Institute (CLSI) guidelines [6]. *P. aeruginosa* ATCC 27853 was used as the quality control strain in antimicrobial susceptibility testing. MBL Etest strips (AB-Biodisk, Solna, Sweden) were used according to the protocol recommended by manufacturer. The MIC was read directly from the test strip at the point where the elliptical zone of inhibition intersected the MIC scale on the strip. The criterion for considering the test positive for MBLs was MIC ratios (MIC of Imipenem alone/MIC of Imipenem plus EDTA) of ≥ 8 . Interpretation was made in accordance with CLSI guidelines [7]. The double disk synergy test (DDST) was performed according to the method of [3]. Three microliters of 2-6 mercaptopropionic acid (Sigma, USA) was applied onto a blank disk and placed 10-15 mm apart from the edge of an imipenem disk. After an overnight incubation, the bacterial isolates with enhancement of growth inhibitory zones (synergistic effect) between both disks were considered as MBL producers [3].

2.3. Duplex PCR assay for detection of *bla*_{VIM, IMP} genes

Bacterial DNA extraction was performed in accordance to the protocol provided by QIAGEN Mini Amp Kit (Qiagen, USA). Duplex PCR assay was carried out for detection of IMP and VIM-type MBLs using the IMP and VIM type primers [1]. Amplification was performed in a 25 μ l mixture containing 1X PCR buffer, 2 mM MgCl₂, 0.2 mM dNTP (MBI Fermentas, USA), 2.5 U *Taq* DNA polymerase (MBI Fermentas, USA) and 2 μ l of DNA template. The PCR conditions were as follows: initial denaturation at 94°C for 5 min; 36 cycles of 94°C for 30s, 55°C 30s, and 72°C for 1:5 min followed by a final extension for 10 min at 72°C. *Ps aeruginosa* strain *bla*_{IMP-7} 55699 used in a previous study [9] and *Acinetobacter* spp. YMC 05/4/P488 *bla*_{IMP-1} and *P. aeruginosa* YMC 95/1/704 for *bla*_{VIM-2} from Korea were used as the positive controls for amplification of *bla*_{IMP} and *bla*_{VIM}, respectively. Nucleotide sequences were analyzed and compared by use of the BLAST computer program (National Center for Biotechnology Information <http://www.ncbi.nlm.nih.gov>).

2.4. Determination of integron class using PCR/RFLP

PCR-RFLP was performed using the degenerate primers hep35 and hep36 to identify the presence of integrons in the isolates [10]. The PCR products were further restricted using *Hinf*I (MBI, Fermentas) to determine the class of integrons.

2.5. Amplification and sequencing of gene cassette regions

The gene cassettes of the class 1 integron were amplified using specific primers for 5' and 3' conserved segments (5'CS and 3'CS) combining with primers VIM-R/IMP-R and VIM-F/IMP-F respectively [11]. Amplification was performed in a 25 μ l mixture containing 1X PCR buffer, 2 mM MgCl₂, 0.2 mM dNTP (MBI Fermentas, Vilnius, Lithuania), 2.5 U *Taq* DNA polymerase (MBI Fermentas, USA) and 2 μ l of boiled bacteria extracts in a Bio-Rad PCR system thermal cycler PCR was carried out using the following condition: an initial denaturation step for 5 min at 95°C and 30 cycles of 1 min at 94°C, 1 min at either 61°C (for VIM-F/3'CS), 57 °C (for VIM-R/5'CS), 60°C (for IMP-F/3'CS) or 57 °C (for IMP-R/5'CS), 5 min at 72 °C, followed by 10 min at 72 °C.

2.6. Sequence analysis of genes encoding MBL gene cassettes in class 1 integrons

Amplified products were purified using PCR purification kit (Geneall, Korea) and sequences were determined using an automated DNA sequencer (ABI prism DNA sequencer, Perkin Elmer ABI, Wellesley, MA) by dideoxy chain termination method. Nucleotide sequences were analyzed and compared by using BLAST software (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>).

2.7. Molecular typing of imipenem-resistant *P. aeruginosa* isolates using random amplification polymorphic DNA analysis (RAPD)

Fingerprinting patterns were generated from random amplification of polymorphic DNA (RAPD) analysis of the isolates, using primer 272 [12]. The RAPD mixture (25 μ l) was composed of 100ng of genomic DNA, 0.5 μ M primer, 1.25U of *Taq* polymerase (MBI Fermentas), 0.2mM of each deoxynucleoside triphosphate (Vivantis. USA), (1X) *Taq* buffer with KCl (pH8) and (2mM) MgCl₂. The following PCR conditions were used: (i) 4cycles, with each cycle consisting of 5min of 94°C, 5min at 36°C and 5min at 72°C and (ii) 30 cycles with each cycle consisting of 1min at 94°C, 1min at 36°C and 2min at 72°C followed by final extension step at 72°C for 10min. The RAPD fingerprinting patterns were analysed by Gel Compare II V_{4.0} software package (Applied Maths, Kortrijk, Belgium).

3. Results

Among the 90 *P. aeruginosa* IRPA isolates used in this study, most resistance was observed towards ceftazidime (57 isolates, 63%) and least resistance towards netilmicin (44 isolates, 48%) (Table 1). Of the 90 IRPA isolates, 32 isolates were identified as MBL-positive using MBL Etest and 34 were positive by double-disk synergy test (DDST).

In addition, 32 of these isolates were found to be multidrug-resistant, conferring resistance to at least six antibiotics (Table 2). All three selected MBL phenotypic assays methods (CDT, DDST, and IP/IPI *E*-test) evaluated were shown to have a sensitivity of 100%. However, specificity of these phenotypic assays was found to differ. Of the three methods, DDST demonstrated the highest specificity (96.6%), followed by IP/IPI *E*-test with the single criteria of IP/IPI ≥ 8 as positive (62.1%). CDT was found to demonstrate the lowest specificity (43.1%). In all three phenotypic assays, false positive MBL producers were detected. These false-positive results may be caused by the presence of unknown and weaker β -lactamases, which is worth further investigation.

Significant differences ($P < 0.001$) were found between the MBL indexes of CDT and IP/IPI *E*-test compared to the direct detection of MBL genes by PCR. However, distinct subsets were evident only with IP/IPI *E*-test between IRPA with and without MBL genes. The high false-positive reporting rate attributed to CDT is not surprising as the isolates tested in this study were IPM resistant, and, therefore, the inhibition zone of ≥ 7 mm in zone diameter in the presence of EDTA may not be considered as a definitive clear cut off criterion to differentiate between MBL-producing and non-MBL-producing IPRA isolates.

Duplex PCR detection identified 32 out of the 90 IRPA clinical isolates as MBL producers. *bla*_{IMP} and *bla*_{VIM} were present in 14 and 18 of the 32 MBL PCR-positive isolates, respectively (Table 3, Figure 1). From the years 2005 to 2006, 12 isolates were identified as MBL-PCR positive and this number increased to 20 isolates in the following years 2007 to 2008 (Table 3). Thirty two of the 34 DDST-positive isolates were tested positive for the presence of MBL genes by PCR and the remaining 2 were tested negative. Although the 2 isolates were MBL PCR-negative, it may probably produce other class of carbapenemases but not the class B MBLs and therefore not detected by the PCR assay. The sequence analysis results have been summarized in Table 3 whereby IMP-4 and VIM-11 were detected at lower frequencies, i.e., two IMP-4 and one VIM-11 in years 2005 to 2006 and none in the following years 2007 to 2008. The IMP-7 and VIM-2 was the most prominent IMP and VIM subtypes identified amongst the Malaysian isolates.

Table 1 Antibiotic susceptibility of 90 imipenem-resistant *P. aeruginosa* isolates Collected from 2005-2008

Antibiotic	Antibiotic susceptibility of Imipenem-Resistant (n=90) <i>P. aeruginosa</i> Isolates (IRPA) from 2005-2008						
	IRPA (n=90) (%) ^a	2005-2006			2007-2008		
		R(n)	In(n)	S(n)	R(n)	In(n)	S(n)
Ciprofloxacin	46(51)	4	-	20	42	-	24
Piperacillin	55(61)	10	-	14	45	-	21
Gentamicin	48(53)	4	-	20	44	-	22
Ceftazidime	57(63)	10	1	13	46	2	20
Amikacin	56(62)	10	-	14	46	-	22
Netilmicin	44(48)	6	-	18	38	-	28
Cefoperazone	51(56)	9	-	15	40	2	24

R: Resistant, I: Intermediate, S: Sensitive

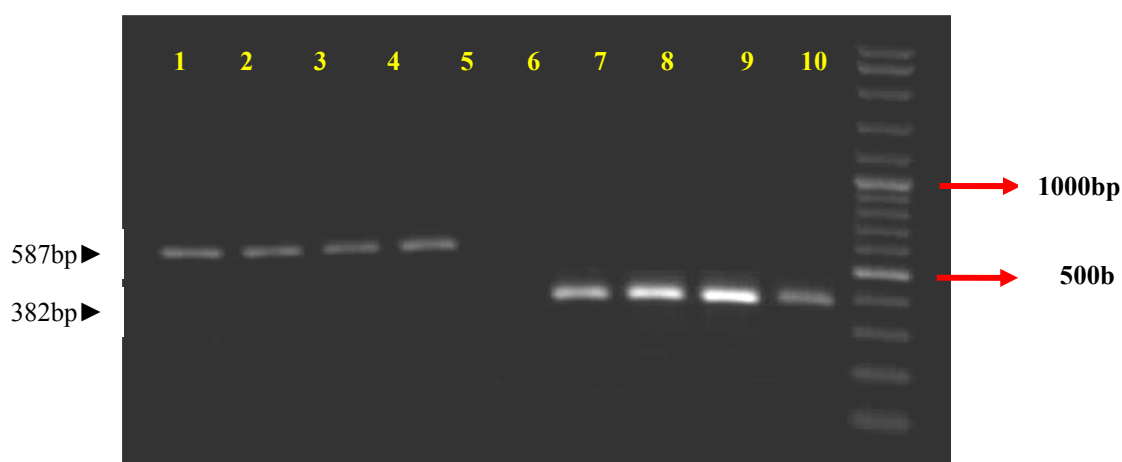
Table 2 Comparison of antibiotic susceptibility of 32 multi-drug resistance strains *P. aeruginosa* during three years of study (2005-2008)

Antibiotic	Antibiotic susceptibility of MDR Imipenem-Resistant <i>P. aeruginosa</i> Isolates (IRPA) from 2005-2008					
	No (n=12) 37%			No (n=20) 62%		
	2005-2006			2007-2008		
	R(n)	In(n)	S(n)	R(n)	In(n)	S(n)
Ciprofloxacin	8	1	3	13	-	7
Piperacillin	12	-	-	20	-	-
Gentamicin	12	-	-	19	-	1
Ceftazidime	11	1	-	13	-	7
Amikacin	10	2	2	13	-	7
Netilmicin	12	-	-	10	1	8
Cefoperazone	11	1	-	18	-	2

R: Resistant; IN: Intermediate; S: Sensitive

Table 3 Detection of metallo- β -lactamase (MBL) gene variants in Imipenem-Resistant *P. aeruginosa* (IRPA) clinical isolates from UMMC

Gene		Imipenem-resistant <i>P. aeruginosa</i> isolates (n=90)	
		2005-2006 ^a (n=28)	2007-2008 ^a (n=62)
<i>bla</i> _{IMP} (n=14)	<i>bla</i> _{IMP-7}	2	10
	<i>bla</i> _{IMP-4}	2	-
<i>bla</i> _{VIM} (n=18)	<i>bla</i> _{VIM-2}	7	10
	<i>bla</i> _{VIM-11}	1	-
MBL Gene PCR-Positive (n=32)		12	20

^aYear of Isolation**Fig. 1** Agarose gel electrophoretic analysis of PCR products generated from amplification of *bla*_{IMP, VIM} genes from *P. aeruginosa*. Lane 1: *bla*_{IMP-7} (strain Ps18), Lane 2: *bla*_{IMP-4} (strain Ps3), Lane 3: IMP positive control (Malaysia 5699), Lane 4: IMP positive control (Korea YMC 05/4/P488), Lane 5: Negative control (distilled water), Lane 6: *bla*_{VIM-2} (strain Ps7), Lane 7: *bla*_{VIM-2} (strain Ps8), Lane 8: *bla*_{VIM-11} (strain Ps23) Lane 9: positive control Korea YMC 95/1/704), Lane 10: 100bp ladder (MBI Fermentas)

4. Detection and class distribution of integrons in *P.aeruginosa*

The presence of integrons was confirmed in 57 (63 %) of the isolates, of which 54 (94.7 %) and three (5.3 %) were identified as class 1 and class 2 integrons, respectively. No class 3 integron was detected in this study. All 32 MBL producing isolates were found to have class 1 integrons.

5. Characteristics of class 1 integron-associated MBL genes

Upon sequencing, seven different Class 1 integron cassette regions were identified from the PCR product using a combination of primers 5'CS with VIM-R/IMP-R and 3'CS with VIM-F/IMP-F. These cassettes include genes encoding resistance to β -lactam antibiotics (*bla*_{IMP-4}, *bla*_{IMP-7}, *bla*_{VIM-2} and *bla*_{VIM-11}) and aminoglycosides (*aadA6*, *accC1* and *aacA7*). Partial fragments of *aacA7* and *bla*_{VIM-2} were detected in the 1115 bp amplicons of a class 1 integron harbouring *bla*_{VIM-2} (GenBank accession no. GU299868). Partial fragments of *accC1* and *bla*_{IMP-7} genes were identified in the 806 bp amplicon of a class 1 integron harbouring *bla*_{IMP-7} (GenBank accession no. GU213192). Sequence analysis of the 656 bp amplicons derived from two *bla*_{IMP-4} isolates revealed the presence of the gene *aadA6* (GenBank accession no. GU213193). A 674 bp amplicon was obtained from the single isolate of *P. aeruginosa* carrying *bla*_{VIM-11}. No gene other than *bla*_{VIM-11} was detected in this isolate (GenBank accession no. GU213191); this result has been published elsewhere [1].

6. Molecular typing of IRPA strains by RAPD

RAPD analysis demonstrated 14 PCR fingerprint patterns generated for the 32 isolates carrying a class 1 integron encoding MBL genes (35.5%). However, 20 PCR fingerprint patterns were generated for the 25 non-MBL isolates carrying class 1 and 2 integrons (28.9 %). Based on the numbers of band differences, two clusters (A and B) were identified in the MBL gene-positive isolates, all carrying class 1 integrons, and two clusters (C and D) in the non-MBL isolates. Cluster A of the MBL isolates comprised 18 (56.25%) isolates carrying *bla*_{VIM} genes, whereas cluster B comprised 14 (43.75%) isolates carrying *bla*_{IMP} genes. The non-MBL isolates were divided into clusters C and D. Cluster C comprised 22 non- MBL isolates carrying class 1 integron and cluster D contained three isolates carrying class 2 integrons. The fingerprinting patterns of cluster D isolates did not show any similarity to the other three clusters (Fig. 2).

7. Discussion

In this study, we demonstrated that DDST was more specific in detecting MBLs in comparison to the CDT as also previously described by Lee (2003) and Pitout (2007) demonstrated CDT as the best method for screening for MBL in *P. aeruginosa* from China and this may be attributed to the differences in population structure of MBL genes between the different geographical areas. Unlike DDST, which is qualitative, CDT and IP/IPI *E*-test are both semiquantitative in nature and enabled the calculation of an MBL index. However, EDTA used in the CDT has membrane-permeabilising properties and could exert a deleterious effect on *P. aeruginosa*; thus resulting in false-positive detection. As such, the MBL IP/IPI *E*-test which exhibited 100% accuracy in the detection of MBL production and is also in accordance with our previous PCR findings is most preferable.

In the present study, 32 MBL PCR-positive genes 14 IMP [*bla*_{IMP-4} (n=2), *bla*_{IMP-7} (n=12)] and 18 VIM [*bla*_{VIM-2} (n=17), *bla*_{VIM-11}, (n=1)] were successfully identified. The first report of IMP-7 in *P. aeruginosa* was described from Canada and Malaysia and more recently from Singapore and Japan [13]. IMP-4 in *P. aeruginosa* was first described in Australia and in the year 2007, IMP-4-producing *P. aeruginosa* was isolated from a French patient repatriated from Malaysia [2]. Only two IMP-4-positive isolates identified in this study and the detection in a French patient admitted in Malaysian hospital suggests the spread of IMP-4 in this region via international travelers. The VIM-2 is the most ubiquitous type of MBLs reported worldwide [14]. Unlike VIM-2, the VIM-11 has been reported in only few countries, including Argentina Italy (*bla*_{VIM-11}, GenBank accession no. AY635904), Taiwan and India) [14]. We identified only a single isolate carrying *bla*_{VIM-11} in this study. Further studies are needed to determine other resistance mechanisms of *P. aeruginosa* to imipenem.

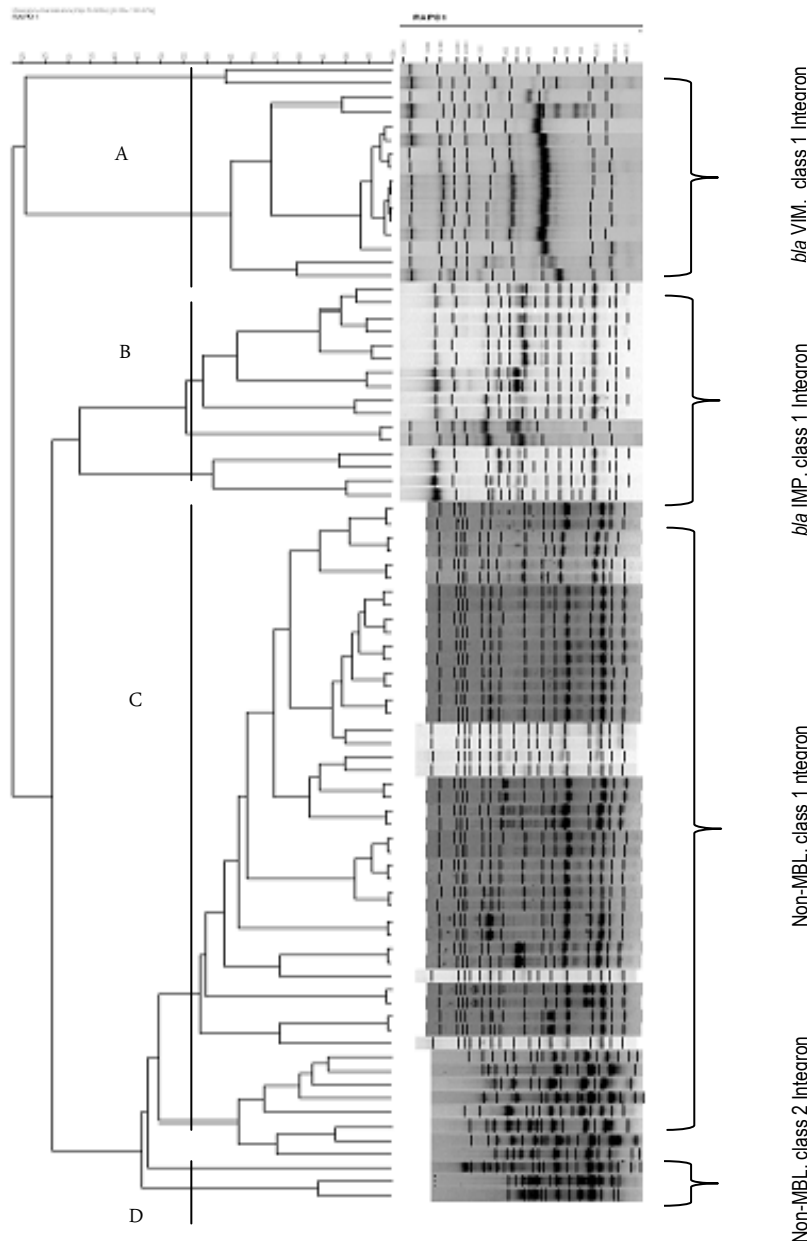


Fig. 2 Dendrogram showing the cluster analysis of *P. aeruginosa* based on primer 272 PCR fingerprinting patterns

In this study, we also confirmed the presence of class 1 integrons in 54 IRPA (32 MBL gene-positive and 22 non-MBL gene) clinical isolates in the UMMC. A previous study at the same hospital demonstrated only one of the 50 IRPA clinical isolates carried the MBL gene and harboured a class 1 integron [9]. Additionally, in 2005, the presence of class 1 integrons was also reported in 79.5% of imipenem-resistant *Acinetobacter baumannii* isolates from Malaysia, with two of them having MBL genes. The increase in Class 1 integrons is not surprising as rapid emergence of bacteria with class 1 integrons of MBL have been reported worldwide [12]. In addition to the class 1 integrons, we also identified class 2 integrons in three of our isolates, which, to the best of our knowledge, is the first report of class 2 integrons in *P. aeruginosa*. Normally, class 2 integrons are most frequently associated with members of *Enterobacteriaceae*, and are also commonly found in *A. baumannii* and *Burkholderia cepacia* [15].

In our study, 12 isolates were found to contain the gene cassettes *accC1* and *bla_{IMP-7}* at the 3'CS of the class 1 integron. An integron with a similar structure has been reported in a *P. aeruginosa* isolate from the neighbouring country Singapore and also in Japan [16]. Thus, we suggest that the class 1 integron is the most abundant integron type present among the clinical isolates in this hospital. Additionally, in this study, 17 isolates were found to contain *aacA7* and *bla_{VIM-2}* at the 5'CS of the class 1 integron. This result is consistent with other findings in which the integron carrying *aacA7* and *bla_{VIM-2}* at the 5' end did not possess the 3'CS [17]. Similar results have been reported in *P. aeruginosa* isolates (strain RON-1) from different countries [11]. However, the downstream regions of the *bla_{VIM-2}* gene of these isolates were found to be different when compared with the *P. aeruginosa* isolate used in our study. The two

isolates with *bla*_{IMP-4} among 14 isolates with *bla*_{IMP} [1] were demonstrated to be harboured by the class 1 integrons that also carried the additional gene cassette *aadA6* conferring aminoglycoside resistance.

Clonality study showed that all MBL producers harbouring class 1 integrons could be divided into two clusters, A and B, whilst the non-MBL producers could be divided into clusters C and D. Strains with the same antibiotic susceptibility demonstrated similar RAPD patterns. Thus, it could be suggested that isolates carrying MBL genes may be detected in different clusters from the non-MBL isolates. Similar to our findings, Pitout et al. (2007) also suggested three clusters for *P. aeruginosa* isolates: cluster 1 (*bla*_{VIM} producing), cluster 2 (*bla*_{IMP} producing) and cluster 3 (non-MBL).

Our findings suggest that MBL genotypes are not homogenous in geographical distribution and as such, generalized criteria for interpretation of MBL phenotypic assays may not be possible. Thus, it is recommended that the phenotypic assays should be assessed and adopted based on the local situation. It is also suggested that PCR-RAPD can act as a cheaper and easier method for routine investigation of the clonality of isolates compared to other methods such as PFGE. Diversity of RAPD types identified in this study suggests that the carriage of the *bla*_{VIM} and *bla*_{IMP} genes by integrons [12] may facilitate the spread of *bla*_{VIM} and *bla*_{IMP} genes among genetically distinct *P. aeruginosa* strains. To the best of our knowledge, this is the first report on the detection of cassette arrays of class 1 integrons (*bla*_{IMP-7} and *accC1*, *aacA7* and *bla*_{VIM-2}, and *aadA6*) from IRPA clinical isolates in Malaysia. This study highlights the resistance to imipenem due to IMP- and VIM-producing *P. aeruginosa* and their associated class 1 integrons. Horizontal dissemination of the class 1 integron-associated MBL genes may contribute to the further emergence of carbapenem resistance in other Gram-negative bacteria. Therefore, appropriate surveillance and control measures are essential to prevent the further spread of MBL-producing organisms in hospitals. Further studies should be carried out to give a better understanding of the impact of integrons on the dissemination of antimicrobial resistance in the clinical settings.

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