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Coexistence of metallo-beta-lactamase-encoding genes in *Pseudomonas aeruginosa*

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***Background & objectives*: The emergence and rapid spread of carbapenem resistance mediated by metallo-beta-lactamase (MBL) in *Pseudomonas aeruginosa* is of major concern due to limited therapeutic options. This study was aimed at detecting the presence of MBL and its association with integrons in imipenem-resistant *P. aeruginosa* isolates and to determine their genetic relatedness.**

***Methods*: A total of 213 *P. aeruginosa* isolates were collected from two tertiary care centres and tested against anti-pseudomonal antibiotics by antimicrobial susceptibility testing, followed by the detection of MBL production by combined disk method. Minimum inhibitory concentration (MIC) of meropenem was determined by E-test. Multiplex polymerase chain reaction (PCR) was performed for the detection of bla_{SPM}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{GIM} and bla_{SIM}. PCR was carried out to characterize the variable region of class 1 integron. Transcongujation assay was carried out for the confirmation of plasmid-mediated resistance. Enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR was performed for determining the genetic relatedness among *P. aeruginosa* isolates.**

***Results*: Of the 213 *P. aeruginosa* isolates, 22 (10%) were found to be carbapenem resistant and these were from pus 18 (82%), urine 2 (9%), sputum 1 (5%) and tracheal wash 1 (5%). Among 22 isolates, 18 (81.8%) were found to be MBL producers by phenotypic method and MIC range of meropenem was 8 to >32 µg/ml. PCR amplification showed that 20 (91%) isolates carried any one of the MBL genes tested: bla_{VIM} and bla_{NDM} in seven (32%) and six (27%) isolates, respectively; bla_{VIM} and bla_{NDM} in three (14%); bla_{IMP} and bla_{NDM} in two (9%); bla_{VIM} and bla_{IMP} in one (5%) isolate. The bla_{VIM}, bla_{IMP} and bla_{NDM} were found to co-exist in one isolate. None of the isolates were positive for bla_{SPM}, bla_{SIM} and bla_{GIM}. All 22 isolates carried class I integron. Of the 20 MBL-positive isolates, transconjugants were obtained for 15 isolates. ERIC-PCR analysis showed all isolates to be clonally independent.**

***Interpretation & conclusions*: Our results showed 10.3 per cent of carbapenem resistance among *P. aeruginosa* isolates, and the coexistence of MBL-encoding genes among *P. aeruginosa* mediated by class I integron.**

Key words Class I integron - ERIC-PCR - metallo-beta-lactamase - *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an important cause of multidrug-resistant (MDR) nosocomial pneumonia, urinary tract infections, surgical site infections and bloodstream infections. It is also the fifth-most common etiological agent of infections, for which carbapenems serve as the last line of drug therapy[1]. The data of the Carbapenem Antimicrobials Pseudomonas Isolate Testing at regional Locations (CAPITAL) surveillance programme in 2010 reported that the overall rates of carbapenem-resistant *P. aeruginosa* ranged from 7.4 to 35.4 per cent[2]. In 2015, a meta-analysis study showed that the rates of carbapenem resistance ranged from 8.7 to 50.4 per cent among *P. aeruginosa*[3]. Carbapenem resistance is mainly due to upregulation of efflux pumps, decreased outer membrane permeability and acquired metallo-beta-lactamases (MBL)[4]. MBL belong to Amber class B, require zinc for its catalytic activity and are inhibited by metal chelators, such as ethylenediaminetetraacetic acid and thiol-based compounds. Various numbers of MBLs are identified worldwide including IMP, VIM, NDM, SPM, GIM and SIM, whereas in India, bla_{NDM}, bla_{VIM} and bla_{IMP} genes are frequently encountered in *P. aeruginosa*[5].

Integrons are mobile genetic elements, usually located in plasmids or transposons. Class I integron are genetic structures capable of capturing gene cassettes, which possess 5'-CS and 3'-CS on either side of the integrated structures. The 5'-CS consists of integrase gene (*Int1*) and attachment site (*attI*), and 3'-CS contains antisepsis resistance *qacED1* gene; the *sul1* gene, which confers resistance to sulphonamides and an open reading frame (ORF) of unknown function, ORF5[6]. MBL-encoding genes are found to be located within the integron structure along with other resistant determinants and rapidly spread among different species by horizontal gene transfer mechanism[7]. Various typing methods such as multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE)[8], ribotying, BOX polymerase chain reaction (PCR), repetitive extragenic palindromic sequence PCR and enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR are available. ERIC-PCR is an easy, inexpensive and reproducible method for DNA typing. Thus, this study was undertaken to detect the presence of MBL and its association with integrons in imipenem-resistant strains of *P. aeruginosa* and to investigate their genetic relatedness using ERIC-PCR.

Material & Methods

A total of 213 non-duplicate isolates of *P. aeruginosa* were collected during a period of November 2013-December 2014 from two tertiary care hospitals in southern India. One hundred and eighteen isolates from the department of Microbiology, Sri Manakula Vinayagar Medical College and Hospital, Puducherry, and 95 isolates from the department of Microbiology, ESIC Hospital, Chennai, were collected. Antimicrobial susceptibility testing was done by Kirby–Bauer disk diffusion method[9] using the following antibiotics (µg): piperacillin (100), piperacillin-tazobactam (100/10), amikacin (30), gentamicin (10), tobramycin (10), netilmicin (30), colistin (10), polymyxin B (300 units), ciprofloxacin (5), ofloxacin (5), levofloxacin (10), ceftazidime (30), cefepime (30), aztreonam (30), ceftazidime-clavulanic acid (30/10), imipenem (10) and meropenem (10) and results were interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines 2013[10]. *P. aeruginosa* ATCC 27853 was included as a standard strain.

Phenotypic detection of carbapenem resistance: MBL production was screened by combined disk method[11]. Briefly, a liquid culture of the test isolate was adjusted to a turbidity of 0.5 McFarland standard and spread on the surface of a Mueller-Hinton agar plate (HiMedia, Mumbai). Two disks of 10 µg imipenem were placed on the agar at a distance of 25 mm apart; 10 µl of 0.5 M EDTA was added to one of the disks. After overnight incubation at 37°C, an increase of 7 mm or more in zone diameter in the presence of imipenem-EDTA as compared to imipenem alone was considered as positive for the presence of an MBL. Minimum inhibitory concentration (MIC) was determined for meropenem using E-strips (HiMedia).

PCR amplification protocols: DNA extraction was done by boiling lysis method[12]. The cell suspension from an overnight culture was boiled at 100°C for 10 min and immediately kept at -20°C for at least 6 h. The supernatant was used as a template for PCR amplification and was stored at -20°C.

The presence of MBL-encoding genes was detected by multiplex PCR using primers specific for bla_{SPM}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{GIM} and bla_{SIM} (Table I)[13,14]. The reaction mixture consisted of 2.5 µl of 10x Taq buffer with $MgCl_2$, 0.5 µl of 10 mM dNTP (2.5 mM each), 0.5 µl of Taq DNA polymerase (1.5 unit), 10 µM of each primer (forward and reverse) in a total volume of 23 µl with 2 µl of DNA. For detection of class 1 integron, integrase gene PCR was performed[15]. The thermal cycling conditions included initial denaturation at 94°C for five minutes followed by thirty cycles of DNA

Table I. List of primers used in this study

Gene	Primer sequence 5'→3'	Expected amplicon size (bp)
IMP	F-GGAATAGAGTGGCTTAAYTCTC R-GGTTTAAYAAAAACAACCACC	232
VIM	F-GATGGTGTTTGGTCGCATA R-CGAATGCGCAGCACCAG	390
GIM	F-TCGACACACCTTGGTCTGAA R-AACTTCCAACTTTGCCATGC	477
SPM	F-AAAATCTGGGTACGCAAACG R-ACATTATCCGCTGGAACAGG	271
SIM	F-TACAAGGGATTCGGCATCG R-TAATGGCCTGTTCCCATGTG	570
NDM	F-GGTTTGGCGATCTGGTTTTC R-CGGAATGGCTCATCACGATC	621
IntI	F-CCTCCCGCACGATGATC R-TCCACGCATCGTCAGGC	280
5'CS	GCCTGTTCGGTTCGTAAGCT	Variable
3'CS	CGGATGTTGCGATTACTTCG	Variable

Source: Refs 13,14

denaturation at 94°C for 30 sec; annealing at 52°C for 40 sec, primer extension at 72°C for 50 sec with a final extension step of 72°C for five minutes (Eppendorf Mastercycler Personal, Hamburg, Germany). BAA 2146 (*Klebsiella pneumoniae*) was used as positive control for bla_{NDM}; NCTC 13476 (*Escherichia coli)* for bla_{IMP} and NCTC 13439 (*Klebsiella pneumonia*) for $bla_{VIM.}$

MBL-encoding genes are located in self-transferable plasmids within integrons; thus, PCR was performed for the variable region in class I integron using 5'-CS and 3'-CS specific primers[16]. The regions located upstream of the *bla* gene were amplified with forward primer of *Int* (5'-CS) gene of class 1 integron together with reverse primer of bla_{VIM} or bla_{IMP} or bla_{NDM} (VIM-R/5'-CS, IMP-R/5'-CS, NDM-R/5'-CS). The sequences located downstream of the *bla* gene were amplified with 3'-CS with reverse-primer of bla_{VIM} or bla_{IMP} or bla_{NDM} (VIM-F/3'-CS, IMP-F/3'-CS, NDM-R/3'-CS).

Conjugation assay: Transfer of resistant genes was evaluated through conjugation assay using a rifampin-resistant mutant of *P. aeruginosa* PU21[17,18]. All MBL-producing isolates were used as donors and *P. aeruginosa* PU21 as the recipient. An overnight culture of donor (0.1 ml) and recipient cells (0.4 ml) were incubated for 18-24 h, and transconjugants grown in trypticase soy agar plates containing rifampin (100 µg/ml) and ceftazidime (4 µg/ml) after 48 h of incubation were selected. Plasmid extraction of donor and transconjugants was done[19] and screened for the presence of carbapenemase-encoding genes by PCR as described above.

Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR): Genomic bacterial DNA was used for the ERIC-PCR reactions using the sequences ERIC 1 (5'-ATGTAAGCTCCTGGGGATTCAC -3') and ERIC 2 (5'-AAGTAAGTGACTGGGGTGAGCG -3'). The reaction mix contained 2.5 µl of 10x Taq buffer with $MgCl_2$, 0.5 µl of 10 mM dNTP (2.5 mM each), 0.5 µl of Taq DNA polymerase (1.5 units), 10 µM of forward and reverse primer each in a total volume of 23 µl with 2 µl of DNA template. PCR amplification was carried out with an initial denaturation of 95°C for seven minutes followed by thirty cycles of DNA denaturation at 94°C for one minute, annealing at 52°C for one minute, primer extension at 65°C for eight minutes and final extension at 65°C for 16 min[20]. ATCC 27853 *P. aeruginosa* was used as control. ERIC-PCR analysis was performed using GelQuest version 3.2.1 (Digital DNA processing, Klein Raden, Germany) and SequentiX (Digital DNA processing) and ClusterVis version 1.8.2 (Digital DNA processing). Pearson Phi coefficient was calculated and compared to evaluate similarity among strains. Clustering was performed by neighbour-joining method.[21]

PCR products were analyzed by electrophoresis with 2% agarose gel in 1x Tris Borate EDTA buffer. The gel was stained with ethidium bromide, and the PCR products were visualized using the gel documentation system (Carestream Gel Logic 212 PRO, New York, USA).

The study protocol was cleared by the Institutional Ethics Committee.

Results

Two hundred and thirteen isolates of *P. aeruginosa* were obtained from various clinical specimens, such as pus (66%), urine (10%), sputum (15%), blood (4%), bronchoalveolar lavage (2%), semen (2%) and tracheal wash (1%).

Among these, 22/213 (10.3%) isolates were resistant to all the antibiotics tested, except colistin and polymyxin B, and were chosen for the present study. Of the 22

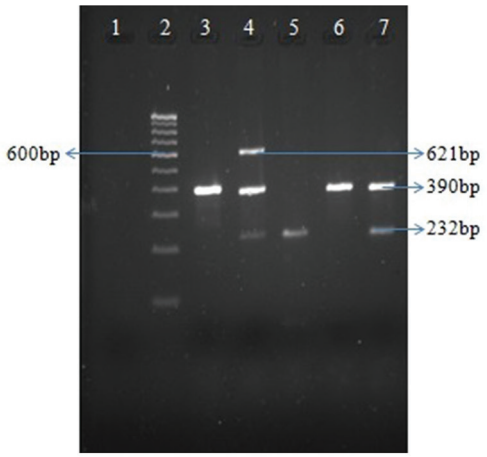

Fig. 1. Multiplex polymerase chain reaction for detection of metallo-beta-lactamase-encoding genes. Lane 1, negative control; lane 2, 100 bp ladder; lane 3, PA32 (VIM positive); lane 4, PA44 (VIM, IMP, NDM positive); lane 5, PAE181 (IMP positive); lane 6, PA27 (VIM positive); lane 7, PAE180 (IMP, VIM positive).

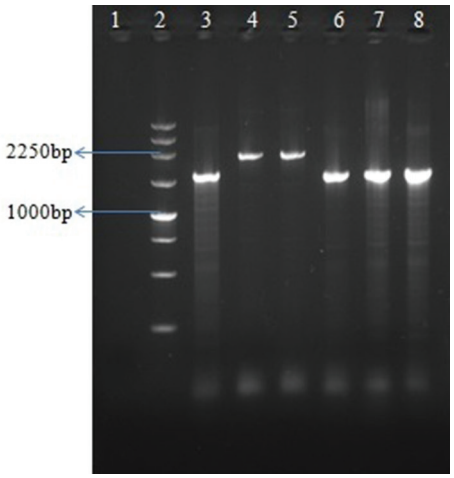

Fig. 2. Polymerase chain reaction for detection of gene cassettes (5'-CS with 3'-CS). Lane 1, negative control; lane 2, 250 bp Ladder; lane 3-8, PA27, PA30, PA42, PA44, PAE120, PAE131.

carbapenem-resistant *P. aeruginosa* (CRPA) isolates, 18 (82%) were considered as positive and four (18%) isolates were negative for MBL production by combined disk method. These isolates had MIC values to meropenem which ranged from 8 to >32 µg/ml by E-test.

Genotypic detection of MBLs revealed that 91 per cent (20/22) of CRPA isolates harboured at least one MBL gene. Combined disk test was positive for isolates that harboured bla_{IMP}, bla_{VIM} and bla_{NDM} genes. Of the 22 isolates, bla_{VIM} and bla_{NDM} genes were detected in seven (32%) and six (27%) isolates, respectively; three (14%) isolates had both bla_{VIM} and bla_{NDM}; two (9%) isolates had bla_{IMP} and bla_{NDM} and one (5%) isolate carried bla_{VIM} and bla_{IMP} in combination. Three MBLs (bla_{IMP}, bla_{VIM} and bla_{NDM}) were found to co-exist in one isolate (Fig. 1). Two (9%) isolates positive for phenotypic MBL production were found to be negative for all the MBL genes tested. None of the isolates carried bla_{SPM}, bla_{GIM} and bla_{SIM}. The distribution of MBL-encoding genes and their source is given in Table II.

Screening of class I integron revealed that all 22 isolates carried *Int I* gene. Using the 59-CS and 39-CS primers, the variable regions of the integrons were amplified. Further, PCR with class I integron (5' & 3') conserved sequence was amplified in 12 (55%) isolates which showed approximately 1700 and 2250 bp in size (Fig. 2) and were not amplified in 10 (45%) isolates. Among 12 VIM-positive isolates, 5'-CS/VIM-R was amplified in five isolates (amplicon size ~1500 bp) whereas 3'-CS/VIM-F was amplified in four isolates (~1000 bp). Of the four IMP-positive isolates, amplification was observed in two isolates with 5'-CS/IMP-R (~500 bp) and no amplification was observed in 3'-CS/IMP-F. Amplification was observed in all 12 NDM-positive isolates using 5'-CS/NDM-R (~500 bp) and 3'-CS/NDM-F (~400 bp). Transconjugants of PU21 *P. aeruginosa* were obtained for 15 isolates and repeated attempts failed to produce the transconjugants for the remaining isolates. The multiplex PCR which was done on the PU21 transconjugants revealed resistance to the same carbapenems as the donor strain.

Analysis of ERIC-PCR showed four main clusters (A-D). Cluster A and B contained the majority (n=17, 77%) of the isolates. Four isolates (18%) and one isolate (5%) formed cluster C and cluster D, respectively (Fig. 3).

Discussion

Treatment of infections caused by *P. aeruginosa* is challenging due to increase in the prevalence of MDR strains. Carbapenems are the most effective drugs used for the treatment of MDR infections; however, their inappropriate usage has led to the emergence of resistance. In south India, 12 per cent of carbapenem resistance was reported in Karnataka and Vellore[22,23]. Others have reported 10.9 and 16 per cent of carbapenem resistance in Puducherry[24] and Chennai[25], respectively. Mendiratta *et al*[26] have shown 8.6 per cent of imipenem-resistance amongst *P. aeruginosa* in a hospital setup. In the present study, 10.3 per cent of isolates were resistant to imipenem and meropenem.

Table II. Distribution of *bla* genes and their source and minimum inhibitory concentration (MIC) value of carbapenem resistant isolates

Isolate ID	Source	MIC of meropenem (µg/ml)	bla_{VIM}	bla_{IMP}	bla_{NDM}	*Int I*
PA27	Pus	>32	+	-	-	+
PA30	Pus	8	+	-	-	+
PA42	Pus	8	-	-	+	+
PA44	Pus	>32	+	+	+	+
PA46	Pus	12	-	-	+	+
PAE90	Urine	16	+	-	-	+
PAE95	Pus	16	+	-	-	+
PAE105	Tracheal wash	24	-	+	+	+
PAE120	Urine	12	-	+	+	+
PAE129	Pus	24	+	-	+	+
PAE131	Pus	12	+	-	+	+
PAE136	Pus	24	-	-	-	+
PAE151	Pus	>32	-	-	+	+
PAE156	Pus	12	-	-	+	+
PAE160	Pus	>32	-	-	+	+
PAE161	Pus	>32	-	-	+	+
PAE180	Pus	>32	+	+	-	+
PAE133	Pus	24	+	-	-	+
PAE135	Pus	16	+	-	-	+
PAE140	Sputum	>32	-	-	-	+
PAE179	Pus	24	+	-	+	+
PAE181	Pus	>32	+	-	-	+

+, Positive; -, Negative

Various phenotypic methods have been described for the detection of MBL. Lee *et al*[27] have shown 100 and 88 per cent sensitivity and specificity in modified Hodge test and 100 per cent sensitivity and specificity for EDTA disk synergy test. A similar finding has been described by Noyal *et al*[28]. In this study, 18 isolates were found to be MBL producers by combined disk method and four isolates which were negative by phenotypic method harboured IMP and VIM genes. Amudhan *et al*[29] have reported 51.4 per cent of carbapenem resistance due to bla_{VIM} /bla_{IMP} in *P. aeruginosa*. Another study reported five types of VIM enzymes in *Pseudomonas* spp[30]. Further, a prospective study conducted in Puducherry showed 13.3 per cent of VIM-2 and 1.3 per cent of IMP-1 type MBL producing isolates of *P. aeruginosa*[31]. In the present study, VIM and NDM genes were detected in 55 per cent and IMP in 18 per cent of CRPA isolates. Two isolates which were positive by phenotypic method were negative with PCR. This may be due to the contribution of integron-mediated ESBLs with carbapenem hydrolyzing activity, increased expression of efflux systems, reduced porin expression and increased chromosomal cephalosporinase activity[32]. Class I integron is the most frequently observed type of integrons. In our study all imipenem resistant isolates carried class I integron. The class I integron conserved sequence was not observed in 10 of 22 isolates and subsequent amplification of the 3'-CS with combination of primers also failed. This showed the lack of 3'CS in class I integron and this may be due to the recombination of 5′-CS of class 1 integron with the *tns* module of class 2. This module is similar to *Tn402* (*TN5090*), progenitor of class I integron[16]. Epidemiological typing by ERIC-PCR demonstrated the presence of four clusters among MDR isolates with majority of isolates falling into cluster A and B. ERIC-PCR profile did not show any genetic relatedness and it indicated individual origins of dissemination. The study has certain lacunae such as lack of clinical details and patient follow up for assessment of clinical outcomes.

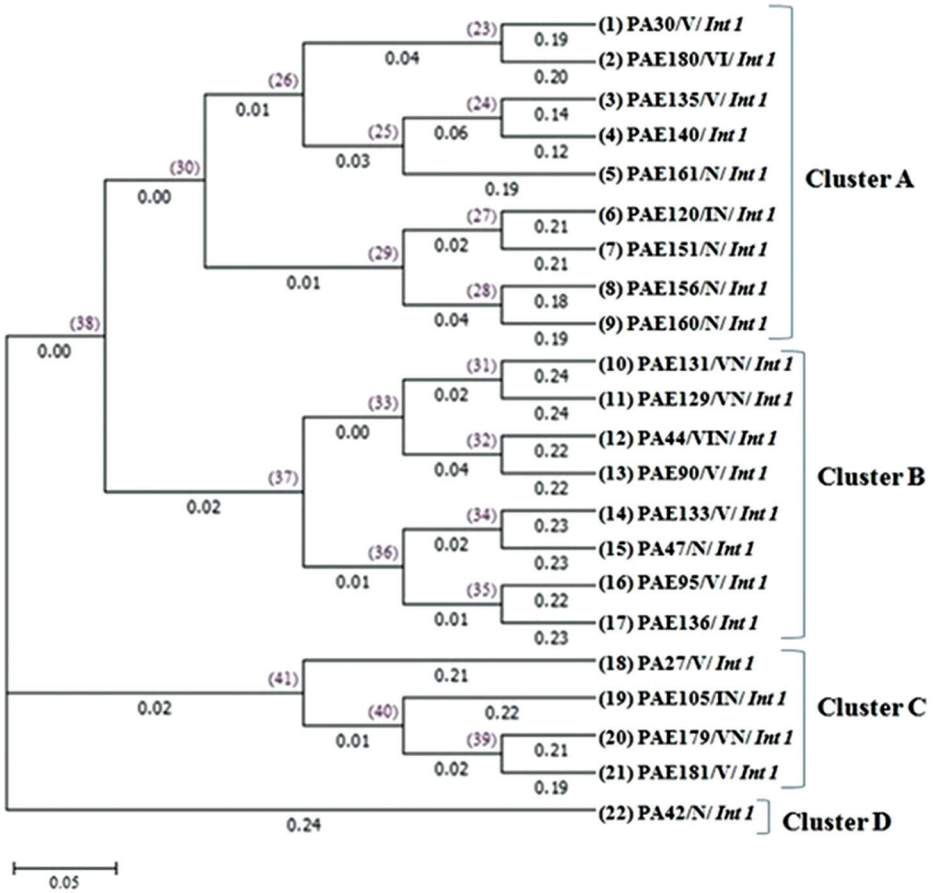

Fig. 3. Dendrogram tree of unweighted pair group method with arithmetic mean method of enterobacterial repetitive intergenic consensus sequence-polymerase chain reaction (ERIC-PCR). The values on top of the horizontal lines represent the node identity and the values given below the horizontal lines represent the branch length. The bar at the bottom of the figure represents an amount of genetic change (0.05).

In conclusion, our findings showed 10.3 per cent of carbapenem resistance among *P. aeruginosa* isolates. Combined disk method was found to be less reliable than genotypic methods for presumptive identification of MBLs. All resistant isolates were associated with class I integron. Our study also documented the coexistence of *bla* MBL genes.

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***Conflicts of Interest*:** None.

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