Occurrence of co-existing $bla_{VIM-2}$ and $bla_{NDM-1}$ in clinical isolates of *Pseudomonas aeruginosa* from India

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**Abstract**

**Background:** $bla_{VIM-2}$ harboring *Pseudomonas aeruginosa* has been reported worldwide and considered as the most prevalent metallo-β-lactamase after NDM which are found horizontally transferable and mostly associated with integron gene cassettes. The present study investigates the genetic background, transmission dynamics as well as stability of $bla_{VIM-2}$ in clinical isolates of *P. aeruginosa* harbor $bla_{NDM-1}$ as well which were collected from October 2012 to September 2013.

**Methods:** Two *P. aeruginosa* strains harboring $bla_{VIM-2}$ along with $bla_{NDM-1}$ were isolated from Silchar Medical College and Hospital, India. Genetic environment of these resistance determinants was determined and transferability was checked by transformation and conjugation assay which was further confirmed by Southern hybridization. Replicon typing was performed to determine the incompatibility group of the resistant plasmid and their stability was checked by serial passage method. Antimicrobial susceptibility pattern of the isolates was determined and their clonal relatedness was checked by pulsed field gel electrophoresis.

**Results:** $bla_{VIM-2}$ was found to be horizontally transferable through an Inc F type plasmid of approximately 30 kb in size. $bla_{VIM-2}$ was found to be associated with integron gene cassette and was flanked by two different types of cassette arrays. Both the isolates were co-harboring $bla_{NDM-1}$ which was carried within Inc N type of plasmid with an approximate 24 kb in size and associated with ISAbA125 in their upstream region. Reduced susceptibility rate as well as high MIC range was observed in case of wild strains and transformants carrying $bla_{VIM-2}$ and $bla_{NDM-1}$.

**Conclusions:** The detection of this co-existence of multiple carbapenem resistance genes in this part of world is worrisome and further investigation is required in order to trace the source and to initiate proper treatment option.

**Keywords:** $bla_{VIM-2}$, $bla_{NDM-1}$, Integron, Inc F type, *Pseudomonas aeruginosa*

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**Background**

Carbapenems are considered as the last drug of choice for most of the serious infections caused by Gram negative bacteria, but due to the prevalence of multidrug resistant organisms these lifesaving drugs were compromised in treating the patients with severe illness. Gram negative bacteria have been documented as the most serious threat in the management of acute infections, as of exhibiting resistance to antibiotics due to the production of carbapenemase enzyme especially metallo-β-lactamase [1, 2]. *Pseudomonas aeruginosa* is one of the most common nosocomial pathogen causing acute infections and frequently reported for carbapenem resistance. VIM metallo-β-lactamase, the second most predominant MBL type responsible for antimicrobial resistance, is pandemic and becoming a health hazard. Among the 37 variant of VIM enzymes, $bla_{VIM-2}$ has now been spread as prevalent MBL among *P. aeruginosa* in all European countries [3] whereas $bla_{NDM-1}$ and $bla_{VIM-18}$ has known to be originated from India [4, 5] where irrational use of
antibiotic is a major contributor for their emergence [6]. VIM and NDM type of MBLs are horizontally transferable and found to be associated with mobile genetic elements, however few molecular information and genetic context is available from this part of India. The potential for quick and extensive dissemination rate of these resistance genes make over a great concern. The present study describes genetic background, transmission dynamics as well as stability of bla\textsubscript{VIM-2} and bla\textsubscript{NDM-1} in clinical isolates of \textit{P. aeruginosa}.

**Methods**

**Bacterial collection**

From October 2012 to September 2013, a total of seventeen \textit{P. aeruginosa} with reduced susceptibility to at least a single carbapenem were collected from Silchar Medical College and Hospital, Silchar. The isolates were then identified by standard biochemical reactions, cytochrome oxidase activity, citrate utilization, pigment production and growth on Cetrimide agar [7].

**Characterization of carbapenemases**

For detection of carbapenemase production, isolates were subjected to modified Hodge test (MHT) and imipenem-EDTA disc test for metallo-\(\beta\)-lactamase production [4]. MHT uses \textit{Escherichia coli} ATCC 25922 as an indicator organism. The presence of clover leaf like indentation in MHT was interpreted as a positive result for carbapenem hydrolysis.

**Molecular characterization of bla\textsubscript{VIM} and bla\textsubscript{NDM} gene**

PCR assay was performed for detection of bla\textsubscript{VIM} as described earlier [8] as well as other metallo-\(\beta\)-lactamase gene (bla\textsubscript{NDM}, bla\textsubscript{IMP}, bla\textsubscript{GIM}, bla\textsubscript{SIM}, bla\textsubscript{SMB}) [4] and amplified products were further sequenced to confirm the presence of resistant gene.

**Detection of co-existing of ESBL genes**

Co-existence of ESBL genes were determined by multiplex PCR targeting bla\textsubscript{TEM}, bla\textsubscript{PER}, bla\textsubscript{OXA-2}, bla\textsubscript{SHV}, bla\textsubscript{CTX-M}, bla\textsubscript{VER} and bla\textsubscript{GES} [9]. Reactions were performed under the following conditions: initial denaturation at 94 °C for 5 min, 33 cycles of 94 °C for 35 s, 51 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 7 min. The amplified products were further sequenced to confirm the co-existence of ESBL genes.

**Detection of genetic context of bla\textsubscript{VIM-2}**

Genetic environment of bla\textsubscript{VIM-2} was determined by performing integrase gene PCR [10] for characterizing the class 1 and class 2 integron. The conserved sequences 5′CS and 3′CS flanking the bla\textsubscript{VIM} were determined using two sets of primers 5′CS and the reverse primer of bla\textsubscript{VIM}, 3′CS and forward primer of bla\textsubscript{VIM} as described earlier [10]. Amplified products were sequenced to determine genetic map of bla\textsubscript{VIM-2}. The reaction condition was: initial denaturation at 95 °C for 3 min, 34 cycles at 95 °C for 30 s, 46 °C for 1 min, 72 °C for 3 min and final extension at 72 °C for 7 min. The presence of mobile element like ISCR in each strain was determined by performing PCR using primers ISCR F (5′-RNSBATAGGAADWNAAHMNV-3′) and ISCR R (5′-BNKDTTNWWHHTCTATVSNY-3′).

**Determination of genetic environment of bla\textsubscript{NDM-1}**

Integron carriage was assessed by performing integrase gene PCR for characterizing the class 1 and class 2 integron. PCR reaction conditions followed was described as earlier [10]. The flanking region of bla\textsubscript{NDM} were determined using two sets of primers 5′CS and the reverse primer of bla\textsubscript{NDM}, 3′CS and forward primer of bla\textsubscript{NDM} [10]. The linkage of bla\textsubscript{NDM-1} with insertion sequence IS\textit{Aba1}25 was determined by using forward primer of IS\textit{Aba1}25 (5′GAA ACT GTC GCA CCT CAT GTT TG-3′) and reverse of bla\textsubscript{NDM-1} (5′-GTA GTG CTC AGT GTC GGC AT-3′) [11].

**Plasmid analysis, transformation and conjugation assay**

bla\textsubscript{VIM} positive bacterial isolates were cultured in Luria–Bertani broth (Hi-Media, India) containing 0.25 μg/ml of imipenem. After overnight incubation, plasmids were extracted by using QIAprep Spin Miniprep Kit (Qiagen, Germany). Isolated plasmids were transformed into recipient strain \textit{Escherichia coli} JM107 by heat shock method and transformants were selected on LB agar with 0.25μg/ml of imipenem. Conjugation experiment was carried out using bla\textsubscript{VIM-2} and bla\textsubscript{NDM-1} harboring transformants as donors and a streptomycin resistant \textit{E. coli} recipient strain B (Genex, India), both the donor and recipient cells were cultured in Luria–Bertani Broth (Hi-Media, India) till it reach an O.D. of 0.8–0.9 at A\textsubscript{600}. Cells were mixed at 1:5 donor-to-recipient ratios and transconjugants were selected on imipenem (0.25 μg/ml) and streptomycin (400 μg/ml) agar plates. Additionally conjugation experiment was also tried using \textit{P. aeruginosa} harboring bla\textsubscript{NDM-1} and bla\textsubscript{VIM-2} as donor and \textit{E. coli} strain B as recipient.

**Southern hybridization for detection of transferability**

Southern blotting was performed on agarose gel by in-gel hybridization with the bla\textsubscript{VIM-2} and bla\textsubscript{NDM-1} probe labelled with DIG HIGH PRIME LABELING MIX (Roche, Germany) detection Kit. The digoxigenin-labeled bla\textsubscript{VIM-2} and bla\textsubscript{NDM-1} specific probe was prepared using primers VIM-F, VIM-R, NDM-F and NDM-R. Plasmid DNA from transformants and transconjugants was
separated by PFGE (CHEF DR-III System, Bio-Rad; USA) and transferred to nylon membrane (Hybond N, UK) and then hybridised with prepared bla$_{VIM}$ and bla$_{NDM}$ specific probe. Detection was performed by using an NBT color detection Kit (Roche, Germany).

**PCR based replicon typing**

The incompatibility type of the plasmids encoding bla$_{VIM}$-2 and bla$_{NDM}$-1 were characterized by PCR based replicon typing targeting 18 different replicon types such as FIA, FIB, FIC, HI1, HI2, I1/Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA [12].

**Antibiotic susceptibility and minimum inhibitory concentration (MIC)**

Antimicrobial susceptibility of parent strains as well as transformants was determined by Kirby-Bauer disc diffusion method against β-lactam and non-β-lactam antibiotics (Hi-Media, India). MIC was also determined by agar dilution method for the isolates and the transformants carrying p$^{VIM-2}$ and p$^{NDM-1}$ towards imipenem (Merck, France), meropenem (AstraZeneca, UK), cefepime, aztreonam (Aristo, India), amikacin (Zuche pharmaceuticals, India), gentamicin (Pharmakem, India), ciprofloxacin (Alkem, India) and polymixin-B (Samarth, India) and interpreted as per CLSI guidelines [13].

**Stability of bla$_{VIM}$-2 and bla$_{NDM}$-1**

Stability of bla$_{VIM}$-2 and bla$_{NDM}$-1 gene was determined by serial passage of the isolates as well as of the transformants in 1:1000 ratios without antibiotic pressure [14]. After each passage the test isolates were subjected to phenotypic detection of MBL and further confirmed the presence of bla$_{VIM}$ and bla$_{NDM}$ by PCR assay.

**Pulsed field gel electrophoresis**

bla$_{VIM}$ and bla$_{NDM}$ positive isolates were typed by pulsed field gel electrophoresis where chromosomal DNA was prepared in agarose blocks and digested with restriction enzyme XbaI (Promega, USA). DNA fragments were separated with CHEF-DR III apparatus (Bio-Rad, USA) and the electrophoresis conditions used were for 24 h at 6 V/cm with pulse rate of 10–40 s as described previously [15]. Clonal relatedness within the isolates was determined by comparing the band patterns.

**Ethical approval**

The work was approved by Institutional Ethical committee of Assam University, Silchar vide Reference Number: IEC/AUS/C/2014-001. The authors confirm that participants provided their written informed consent to participate in this study.

**Results**

Two clinical isolates of *P. aeruginosa* (PA-37 and PA-131) harboring bla$_{VIM}$ were recovered from Silchar Medical College and Hospital, India. The gene was further sequenced and confirmed as bla$_{VIM}$-2 variant. The first isolate (PA-37) was recovered from pus samples of a 55 year old female patient suffering from wound infection admitted in surgery ward in December 2012 while the second one (PA-131) was isolated from urine of a 40 years old female patient with UTL who attended gynecology outpatient department (OPD) in the month of February 2013. bla$_{VIM}$-2 was found to be horizontally transferable as the gene could be successfully conjugatively transferred from transformed *E. coli* JM107 to recipient *E. coli* strain B through an Inc F type plasmid having approximate size of 30 kb. These findings were further confirmed by Southern hybridization results. However, conjugative transfer of plasmids from *P. aeruginosa* to *E. coli* was not successful with our experiment. In both the isolates bla$_{VIM}$-2 was located within integron gene cassette and was flanked by other antimicrobial resistant determinant like gene for aminoglycoside resistance (Fig. 1). The two different types of cassette arrays observed were bla$_{VIM}$-2, aadB-dhfrA-orfC-qacE-sul1 (PA-37) and aadB-aacA7-bla$_{VIM}$-2’-dhfrA1-orfC-qacE-sul1 (PA-131). Both the isolates were co-harboring bla$_{NDM}$-1 and further ESBL screening revealed the presence of bla$_{VEB-1}$ gene in these isolates. However, bla$_{NDM}$-1 gene could not be hybridized with 30 kb plasmid that was harboring bla$_{VIM}$-2, but with a 24 kb plasmid which was successfully hybridized with bla$_{NDM}$-1 specific probe (Fig. 2). Linkage of ISAba125 was observed in the upstream region of bla$_{NDM}$-1 whereas no association with integron gene cassette could be established. Interestingly on analyzing the stability of MBL genes i.e. bla$_{VIM}$-2 and bla$_{NDM}$-1 in case of wild type isolates these resistant genes were stable even after hundred consecutive passages but among their transformants, complete plasmid was lost after fifty-six passages in case of bla$_{VIM}$-2 while bla$_{NDM}$-1 was retained till ninety passages without any antibiotic pressure. These bla$_{VIM}$-2 positive
isolates showed resistance towards most of the antibiotics including piperacillin/tazobactam, co-trimoxazole, amikacin, gentamicin, netilmicin and quinolone group of drugs. MIC values obtained for both the isolates were above breakpoint towards ciprofloxacin, aminoglycosides, β-lactam-β-lactamase inhibitor as well as to polymixin B (Table 1). The two bla_{VIM-2} harboring isolates of _P. aeruginosa_ were found to be clonally different from each other on the basis of their PFGE banding pattern (Fig. 2).

**Discussion**

It has been evidenced that the rapid emergence and dissemination of carbapenemase producing bacteria in this subcontinent is mainly due to the acquisition of _bla_{NDM-1}_ [4]. But in the present study, we described an additional carbapenem resistance determinant i.e. _bla_{VIM-2}_ which played a significant role in carbapenem resistance. Although _bla_{VIM-2}_ with integron gene cassette is reported in previous studies [8], while in the present study, the genetic context of _bla_{VIM-2}_ underscores their diverse origin and persistence along with other resistant genes. In the present study _bla_{VIM-2}_ was found to be associated with the gene cassette along with other resistance determinants which is in agreement to the reports of Toleman et al. [16]. The association of other resistance determinants along with VIM type MBL confers the phenotype to become resistant to most of the available antimicrobial agents. The study reports the presence of

**Table 1** MIC (μg/ml) of the _bla_{VIM-2}_ and _bla_{NDM-1}_ harboring isolates and their transformants

| Organisms | Imipenem | Meropenem | Cefepime | Aztreonam | Amikacin | Gentamicin | Ciprofloxacin | Piperacillin-tazobactam | Polymixin B |
|-----------|----------|-----------|----------|-----------|----------|------------|---------------|-------------------------|-------------|
| PA-37° | >256     | >256      | >256     | >256     | >256     | 256        | >256          | >256                  | >256        |
| _E. coli_ JM107 (p{VIM-2/37})° | 64       | 32        | 128      | 64        | 64       | 64         | 64             | 64                     | 32          |
| _E. coli_ JM107 (p{NDM-1/37})° | 64       | 64        | 128      | 64        | 128      | 64         | 64             | 64                     | 32          |
| PA-131° | >256     | 128       | >256     | >256     | >256     | 256        | >256          | 256                  | 256         |
| _E. coli_ JM107 (p{VIM-2/131})° | 16       | 16        | 64        | 64       | 64       | 64         | 64             | 64                     | 32          |
| _E. coli_ JM107 (p{NDM-1/131})° | 32       | 16        | 128      | 64        | 64       | 64         | 64             | 64                     | 16          |
| _E. coli_ JM107 | 0.06     | 0.012     | 0.06     | 0.12      | 0.06     | 0.125      | 0.06            | 0.12                   | 0.006       |

*° Donor strain  
°° Recipient _E. coli_ carrying plasmid of _bla_{VIM-2}_  
°°° Recipient _E. coli_ carrying plasmid of _bla_{NDM-1}_.

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**Fig. 2** PFGE and hybridization analysis of _bla_{NDM-1}_.

Lane 1 and 4 The undigested total DNA of PA 37 and PA 131 respectively; Lane 2 and 3 The PA 37 total DNA is digested with S1 and Xba I respectively; Lane 5 and 6 PA 131 total DNA digested with S1 and Xba I respectively; Lane 7 The undigested plasmid of PA 37; Lane 8 and 9 _E. coli_ transconjugant carrying plasmid of PA 37 digested with S1 and Xba I respectively; Lane 10 Hybridized p{PA-37} with probe; Lane 11 The undigested plasmid of PA 131; Lane 12 and 13 _E. coli_ transconjugant carrying plasmid of PA 131 digested with S1 and Xba I respectively; Lane 14 Hybridized p{PR-131} with probe; Lane 15 The total DNA of recipient _E. coli_ without plasmid; Lane 16 and 17 The total DNA of recipient _E. coli_ without plasmid digested with S1 and Xba I respectively.

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| Organisms | Imipenem | Meropenem | Cefepime | Aztreonam | Amikacin | Gentamicin | Ciprofloxacin | Piperacillin-tazobactam | Polymixin B |
|-----------|----------|-----------|----------|-----------|----------|------------|---------------|-------------------------|-------------|
| PA-37° | >256     | >256      | >256     | >256     | >256     | 256        | >256          | >256                  | >256        |
| _E. coli_ JM107 (p{VIM-2/37})° | 64       | 32        | 128      | 64        | 64       | 64         | 64             | 64                     | 32          |
| _E. coli_ JM107 (p{NDM-1/37})° | 64       | 64        | 128      | 64        | 128      | 64         | 64             | 64                     | 32          |
| PA-131° | >256     | 128       | >256     | >256     | >256     | 256        | >256          | 256                  | 256         |
| _E. coli_ JM107 (p{VIM-2/131})° | 16       | 16        | 64        | 64       | 64       | 64         | 64             | 64                     | 32          |
| _E. coli_ JM107 (p{NDM-1/131})° | 32       | 16        | 128      | 64        | 64       | 64         | 64             | 64                     | 16          |
| _E. coli_ JM107 | 0.06     | 0.012     | 0.06     | 0.12      | 0.06     | 0.125      | 0.06            | 0.12                   | 0.006       |
aminoglycosides resistance genes aadB and aacA7 on the same gene cassette along with bla_{VIM-2}′, thus making the phenotype resistance to amikacin and gentamicin as well. In the year 2012, Toleman et al. [11] reported the association of insertion sequence ISAb125 in the upstream region of bla_{NDM-1} in Acinetobacter baumannii, we too observed the same insertion sequence present in the upstream region of bla_{NDM-1} in P. aeruginosa. Presence of NDM-1 in P. aeruginosa was for first time recorded in 2011 from patients in Serbia [17] and the same working group has reported that resistance determinant is chromosomally located in this particular organism [18]. Similarly, a report from India also has established its chromosomal location [19]. However, in our recent study we found presence of bla_{NDM-1} on plasmid DNA indicating a possible shift from one to another genetic location [20]. Future experiments will show which direction (chromosome to plasmid or vice versa) of the transfer took place in the organism. High MIC range as well as reduced susceptibility rate against majority of the tested antibiotics was observed in case of bla_{VIM-2} and bla_{NDM-1} harboring wild strains and transformants of bla_{VIM-2} and bla_{NDM-1}. Earlier studies reported [16] that carbapenemase producing isolates remain susceptible to polymixin B whereas both of our study isolates were found to be resistant to this antibiotic, which could be a challenging situation with no or too limited treatment option. The association of mobile genetic element with bla_{VIM-2} may facilitate their mobilization to other susceptible organisms. On performing the transmission dynamics of the strains and it was evident that bla_{VIM-2} and bla_{NDM-1} was horizontally transferable. It may be noted that in our study, the plasmid of P. aeruginosa encoding the NDM-1 was conjugally transferred from the E. coli transformants to recipient E. coli strain although conjugation of the same plasmid was not successful from original host P. aeruginosa to E. coli. The reason for absence of plasmid conjugation could be due to presence of some physiological barriers within these two strains or we did not find the appropriate laboratory conditions for initiation of conjugation or present plasmid do not carry functional Tra operon in P. aeruginosa. Serial passage of transformants harboring both bla_{VIM-2} and bla_{NDM-1} showed that bla_{NDM-1} gene is more stable compare to bla_{VIM-2}, may be because E. coli is an unnatural host for bla_{VIM-2}. The distinguishable pulsotypes of the two strains along with their different genetic arrangements indicates horizontal acquisition from diverse source and antibiotic pressure in this hospital setting.

Conclusions
Co-existence of multiple carbapenem resistance determinants in hospital isolate is worrisome and a matter of concern for infection control management considering the treatment option and clonal expansion. Thus, the current finding is of epidemiological interest, which requires immediate steps to initiate proper treatment option.

Abbreviations
VIM: Verona integron-encoded MBL; NDM: New Delhi metallo-β-lactamase; MBLs: metallo-β-lactamases, Bla: beta-lactamase; MHT: modified Hodge test; PCR: polymerase chain reaction; SIM: Seoul imipenemase; GIM: German imipenemase; SMB: Serratia metallo-β-lactamase; ESBL: extended spectrum beta-lactamase; 5′ & 3′-conserved segment; ISCR: insertion sequence common region; MIC: minimum inhibitory concentration; PFGE: pulse field gel electrophoresis; UTI: urinary tract infection; CLSI: Clinical and Laboratory Standards Institute.

Authors’ contributions
DP: Design and performed the experimental work, literature search, data collection, analysis and prepared the manuscript. DD: Participated in experiment designing and manuscript correction. AP: Participated in sample collection and part of experiments. SM: Carried out the Southern hybridization experiment and result analysis. AC: Participated in experiment designing and manuscript correction. GDS: Participated in drafting the manuscript. AB: Supervised the research work and participated in designing the study and drafting the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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