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Characterization of phenotypically metallo-β-lactamase positive *Pseudomonas aeruginosa* human isolates from Himachal Pradesh for MBL genes *(bla_{VIM-2} and bla_{IMP-1})*, integrase gene class 1, 2, 3 (*int1, int2 and int3*) and sulphonamide resistance gene (*sul1*)

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Abstract: *Background & objectives:* The present study highlights the occurrence of MBL gene (*bla_{VIM-2}*), integron class 1 (*int1*) and sulphonamide resistance (*sul1*) genes in *P. aeruginosa* isolates recovered from patients of Shimla region of India. *Methods: P. aeruginosa* isolates detected positive for MBL by Combined Disc and Ezy MIC tests were characterized by PCR amplification of segments of *bla_{IMP-1}* and *bla_{VIM-2}*, integrase genes encoding integron classes *int1*, *int2* and *int3*, and *sul1* gene. The nucleotide sequence homologies with the published sequences of *Pseudomonas* spp and other gram-negative bacilli were determined. *Results:* All the confirmed isolates of *P. aeruginosa* were further subjected to nucleotide sequencing of 16SrRNA amplicons. MBL gene (*$bla_{VIM-2)}$* was amplified in six out of 27 MBL positive isolates, *int1* gene in eleven and *sul1* gene in 14 MBL positive isolates .Only one isolate Pa138 carried both *bla_{VIM-2}* and *int1* genes. The specificity was established by nucleotide sequencing of the amplicons. *Interpretation & Conclusion:* The occurrence of *$bla_{VIM-2,}$* *int1* and *sul1* genes has been reported in the *P. aeruginosa* isolates of Shimla region of Himachal Pradesh. The studies might be useful to the clinicians for selecting suitable antibiotic for treating and controlling *P. aeruginosa* infections in this region.

Keywords: *P. aeruginosa*, MBL, integron, *bla_{VIM-2}*, *int1*, *sul1*.

Introduction

P. aeruginosa is an opportunistic, nosocomial pathogen of immuno-compromised individuals such as patients suffering from AIDS, cancer, burn wounds and cystic fibrosis. Infections associated with this bacterium are nosocomial, respiratory tract infections including ventilator-associated pneumonia (VAP), dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections. *P. aeruginosa* is responsible for 11-13.8% of the hospital acquired infections [1] but in ICUs, this percentage is even higher i.e. 13.8-22.6% [2-4].

Due to overzealous and indiscriminate use of antibiotics for treating *P. aeruginosa* infections, multidrug resistant strains of this organism have emerged particularly in hospitals settings. We observed higher proportion (69.50%) of MDR isolates prevalent at Shimla region of Himachal Pradesh [5]. Among the MDRs, 27.55% isolates were found MBL producers. Production of MBLs is probably the major defence mechanism of *P. aeruginosa* against β-lactam antibiotics which degrade this group of antibiotics [6-7]. MBLs or class B beta lactamases are zinc dependent enzymes which are gaining clinical significance because these enzymes hydrolyze almost all β-lactams, with the exception of monobactams. No clinically available inhibitor is known till date [8]. These enzymes are encoded by different MBL genes located as cassettes in integrons that provide them with the potential for expression and dissemination. Generally, the MBL genes are located on class 1 integrons. The present study has been designed to identify and screen for metallo-β-lactamase genes encoding for Imipenamase (IMP) and Verona Imipenemase (VIM) in the highly resistant phenotypes of *P. aeruginosa* recovered from Shimla region of the state of Himachal Pradesh. We present here the results of the occurrence of *bla_{VIM-2}*, integrase gene (*int1*) and *sul1* gene among MBL positive isolates. This would give an insight into the nature of the *P. aeruginosa* isolates prevalent in this region.

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Material and Methods

This study was conducted at Department of Microbiology, Shoolini University, Solan. The research project was undertaken after seeking clearance from the Institute Ethics Committee (IEC) of the University through letter no. SUBMS/IEC/12/46 dated March 19, 2012.

Bacterial isolates: The present study is an extension of our earlier investigation as mentioned above, in which 141 *P. aeruginosa* isolates recovered from human patients at Indira Gandhi Medical College, Shimla, were confirmed as *P. aeruginosa* of which 69.50% isolates were found resistant to multiple antibiotics by *in vitro* antibiotic cultural sensitivity assay. Out of these, 27 *P. aeruginosa* isolates which were positive for MBLs by phenotypic test were utilized in the present study for genotypic characterization.

PCR screening for MBLs, integron classes and sulphonamide resistance genes

Extraction of genomic DNA: The extraction of genomic DNA was carried out as per method of Wilson (1997) [9]. The details of the method are as follows: nutrient broth in 5ml volume in each tube was inoculated with *P. aeruginosa* isolates individually and incubated for 24h at 37°C. Fresh culture in 2ml volume was centrifuged for 10 min to get a compact pellet of the bacteria. The pellet was suspended in 567µl TE buffer. SDS (10%) in 30 µl volume and 3 µl of 20mg/ml proteinase K were then added to give a final concentration of 100µg/ml proteinase K in 0.5% SDS. The contents were thoroughly mixed and incubated for 1 h at 37°C. For precipitation of DNA, 5M NaCl in a volume of 100µl was then added and mixed thoroughly. Cetyl trimethyl ammonium bromide (CTAB)/NaCl solution in 80µl volume was then added to it and the contents mixed thoroughly and incubated at 65°C for 30 min. Equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1) mixture was added to the contents and mixed thoroughly, centrifuged for 10 minutes after through mixing. Aqueous supernatant was removed to a fresh microcentrifuge tube. To this, an equal volume of phenol/chloroform/ isoamyl alcohol was again added and the contents were centrifuged for 10 minutes at 10,000 rpm. The supernatant was transferred to a fresh tube. In order to precipitate the DNA, 600µl of 70% ethanol was added, the tube was shaken back and forth until a stringy white DNA precipitate was clearly visible, which was pelleted by spinning briefly at room temperature. The pellet was re-dissolved in 100 µl of TE buffer and stored at -20°C until its use in the experiment.

PCR amplification: The DNAs extracted from the isolates was subjected to PCR amplification of bla_{IMP-1} and bla_{VIM-2}, integron classes *int1*, *int2* and *int3* and sulphonamide resistance (*sul1)* genes. Primer sequences used in the PCR assays for amplifying the mentioned genes are given in Table 1. The primers were designed with the help of Primer3 tool from the reference sequences of the genes in the GeneBank database.

Table-1: Details of primers used for the detection of carbapenemase genes in this study and their amplicon sizes

Target gene	Primers (5'-3')	Amplicon size	Source/Reference
blaIMP-1	F:ATTGCTACCGCAGCAGAGTC R:TTTCAAGAGTGATGCGTCTCC	764	Present study
blaVIM-2	F:TTTGACCGCGTCTATCATGGC R:CAACGACT GAGC GATTTGTG	856	Present study
int1	F:CCTACCTCTCACTAGTGAGG R:GTGCCTTCATCCGTTTCCAC	927	Present study
int2	F:CACGGATATGCGACAAAAAGGT R:GTAGCAAACGAGTGACGAAATG	789	Present study
int3	F:GCCTCCGGCAGCGACTTTCAG R:ACGGATCTGCCAAACCTGACT	980	Present study
sul1	F:TGGTGACGGTGTTCGGCATTC R:GTTTCCGAGAAGGTGATTGCG	784	Present study

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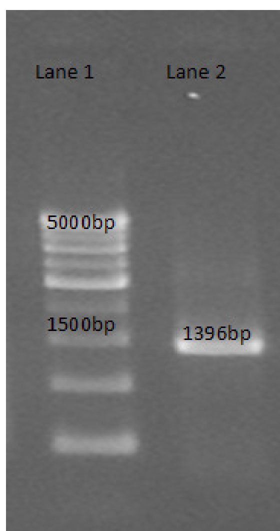
Nucleotide sequencing of amplicons: The amplicons of different targeted genes of selective *P. aeruginosa* isolates were sequenced for their nucleotides, by Molecular Diagnostics and Research Laboratories (MDRL), a commercial facility at Chandigarh. The nucleotide sequence variability in *int1*, *sul1* and & bla_{VIM2} genes of the isolates was detected by multiple sequence alignment using CLUSTAL OMEGA, with the homologous gene sequences obtained by BLAST analysis with the published sequences of National Centre for Biotecnological Information (NCBI).

Results

PCR amplification of 16S rRNA gene in

MBL positive P. aeruginosa isolates: In order to further authenticate the identity of all the 27 *P. aeruginosa* isolates which were detected MBL positive by phenotypic tests, molecular characterization using the amplification of 16S rRNA was carried out. The BLAST analysis of the amplicons revealed 100% sequence homology to published 16S rRNA amplicons of *P. aeruginosa* confirming thereby their identity as *P. aeruginosa*. The results of PCR amplifications of isolate Pa99 on electrophoresis of the PCR products revealed a band of 1400bps (Fig-1). The results of CLUSTAL OMEGA multiple sequence alignment of 16S rRNA gene amplicon of the isolate Pa99 with other reported corresponding sequences of different *P. aeruginosa* isolates revealed 100% homology .

Fig-1: Agarose gel electrophoresis of 16S rRNA amplicon of isolates Pa99 (Lane 2) and 500bp ladder of molecular size of 5kb (Lane 1)



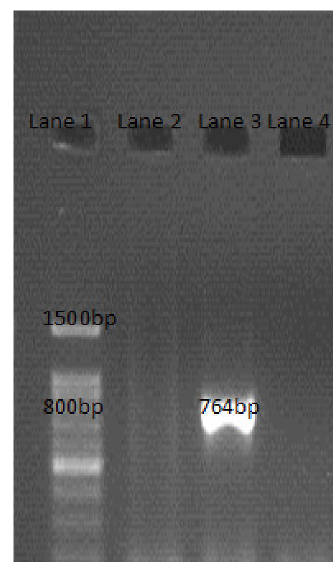
PCR amplification of bla_{VIM-2} gene: bla_{VIM-2} gene was amplified in six out of 27 MBL positive isolates (Table 2). The amplicon size of 750bp on agarose gel electrophoresis was observed (Fig-2). However, the amplification of bla_{IMP-1} gene in any of the isolates was not observed.

Table-2: PCR amplification of genes of MBL positive *P. aeruginosa* (n=27)

Target gene	No. of samples positive in PCR assay
Blavim-2	6
Blaimp-1	0
Int1	11
Int2	0
Int3	0
Sul1	14

Int1-integron1, blavim2-Beta lactamase VIM-2, blaimp1-Beta lactamaseIMP-1, sul1-sulphonamide

Fig-2: Agarose gel electrophoresis of PCR products of VIM-2 gene amplicons of isolate Pa138 (Lane 3). Amplification was not observed in case of isolate no. 147(Lane 2). Lane 1 is loaded with a molecular marker of 1.5kb Consisting of 100bp ladder.



PCR amplification of integrase gene: Int1 gene was amplified in eleven out of 27 isolates (Table 2). The amplicon size of 927bp was observed (Fig 3). However, integrase genes *int2* and *int3* were not amplified in any of the isolate.

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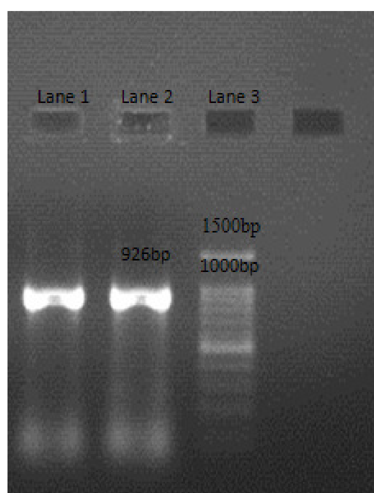
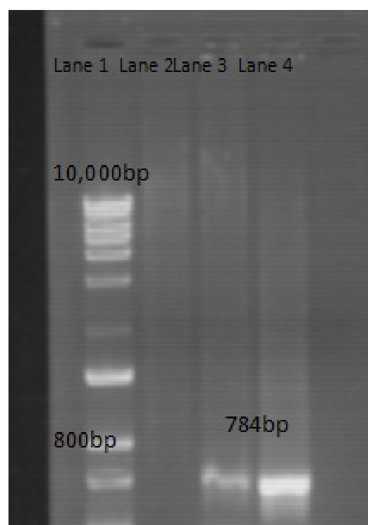
Fig-3: Agarose gel electrophoresis of PCR products of int1 gene amplicons of isolate Pa9 (Lane 1) and Pa138 (Lane 2). A band of 926bp is visible. Lane 3 contains molecular marker of 1.5kb consisting of 100bp ladder.



PCR amplification of sul1 gene: sul1 gene was amplified in fourteen MBL positive isolates (Table 2). Amplicon size of 780bp was observed on agarose gel electrophoresis (Fig 4).

Fig-4: Agarose gel electrophoresis of PCR products of sul1 gene amplicon of isolate Pa91(Lane 3) and Pa9 (Lane 4) and 1kb ladder of molecular size of 10,000bp is loaded in lane 1.



Association of different genes of the isolates: The occurrence of different genes amplified in the study is presented through Table 3. The study revealed that *int1* and *sul1* genes together were present in 9 isolates. bla$_{VIM-2}$ and *sul1* genes were present in two isolates and only one isolate Pa138 carried both bla$_{VIM-2}$ and *int1* genes.

Table-3: Detail of isolates amplified for various genes

Isolate No.	*blavim-2*	*int1*	*sul1*
Pa9	-	+	-
Pa16	+	-	+
Pa57	-	+	+
Pa61	-	+	+
Pa64	-	+	+
Pa91	-	+	+
Pa93	-	+	+
Pa95	-	+	+
Pa97	+	-	-
Pa99	-	+	+
Pa102	-	-	+
Pa103	-	-	+
Pa106	+	-	+
Pa116	+	-	-
Pa123	+	-	-
Pa138	+	+	-
Pa143	-	+	+
Pa144	-	-	+
Pa147	-	+	+
Total	**6**	**11**	**14**

Nucleotide sequencing of amplicons: The specificity of the amplicons was established by nucleotide sequencing of the amplicons of six isolates for *bla$_{VIM-2}$* gene, and three isolates each for *int1* and *sul1* gene amplicons. The BLAST analysis of nucleotide sequences of *bla$_{VIM-2}$* gene amplicon of the isolate (Pa138) revealed 99% sequence homology with *Klebsiella oxytoca, Acinetobacter berezinae, Citrobacter freundii, Pseudomonas sp., Stenotrophomonas maltophilia, Pseudomonas putida, Pseudomonas aeruginosa* and *Acinetobacter baumanii*.

Besides, the nucleotide sequences of the VIM-2 gene amplicon of one of the isolate, Pa16 was compared to the published sequences of other genes in the VIM subgroup 2 i.e. VIM-24 (HM855205.1), VIM-23 (GQ242167.1), VIM-11 (DQ022682.1), VIM-31 (JN566054.1) and VIM-6 (AY165025.1) which had 99-100% homology with our sequence. Such sub-grouping has been done

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by Zhao and Hu (2010) who utilized the amino acid sequences for sub-grouping of VIM gene. A nucleotide sequence homology of 98% was demonstrable on BLAST analysis of *int1* gene amplicon of isolate Pa138 with *Klebsiella oxytoca*, *Proteus mirabilis* and *Escherichia coli.*

The BLAST analysis of sequence of *sul1* gene of isolate Pa57 by CLUSTALW analysis showed 99% homology with *Klebsiella pneumoniae*, *Proteus mirabilis*, *Achromobacter oxylidans*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Escherichia coli*, *Enterobacter cloacae*, *Acinetobacter baumannii* and *Acinetobacter johnsonii*.

Discussion

P. aeruginosa has gained significance as one of the important nosocomial bacterial pathogen. Treatment of infections due to this organism has become very difficult because of the emergence of multi-drug resistant strains in the hospital settings and community. An increasing frequency of multidrug-resistant (MDR) *P. aeruginosa* strains implicated in different disease conditions has been reported from several countries of the world including India. There is thus, an urgent need, to characterize the resistant strains so as to develop suitable therapeutic and other control strategies. The present study has therefore been designed with the objective of characterizing multidrug resistant and metallo-β-lactamase (MBL) producing strains prevalent in the hospital settings of Himachal Pradesh. Such strains pose a threat due to their inter and intra species spread of resistance genes through several mechanisms. Out of over 800 β-lactamases identified from Gram-negative bacilli, at least 120 β-lactamases have been detected in *P. aeruginosa* [10].

In the present study, the amplification of bla_{VIM-2} gene segment was achieved in 6/27 (22.22%) phenotypically MBL positive isolates. We, however, did not achieve amplification of IMP-1 gene in any isolate. These observations suggest that other MBL gene(s) such as NDM-1, GIM-1, SIM-1 etc may be present in such isolates in which IMP-1 gene was not detected by PCR which might be responsible for resistance against carbapenems. It is also possible that mechanisms other than the action of β-lactamase IMP-1 might operate in the development of resistance. For example, it is a well-established fact that EDTA used to detect MBL in phenotypic test may increase bacterial cell-wall permeability and that zinc (chelated by EDTA) accelerates imipenem decomposition and decreases OprD expression of *P. aeruginosa*. Another alternative would be the presence of OXA-10 and/or OXA-14 stabilized in the dimeric form by metal ions (Zn++), as dimeric forms are more active than monomeric form, increasing its enzymatic activity and turning the enzyme more efficient to inactivate carbapenems and other β-lactam antibiotics [11]. Wirth et al (2009) [12] from Brazil and Shahcheraghi et al (2010) [13] from Iran have produced experimental evidence to these observations. In India, other workers have detected bla_{IMP} and bla_{VIM} genes in 59.02% of *P. aeruginosa* isolates in Chennai [14] and 61.1% strains carried bla_{VIM} and 3% carried bla_{IMP} in Tamil Nadu [15]. Our results however, reflect lower frequency of bla_{VIM-2} gene and absence of bla_{IMP-1} gene in the isolates of this region.

On further analysis of the MBL positive phenotypes, for the presence of integron classes, *int1*, *int2* and *int3* by PCR revealed that 11 isolates (40.7%) contained class 1 integrons. However, the amplification was not seen for other integron classes (*int2* and *int3*). The amplification was achieved in case of integron class 1 only in 11 (40.7%). The frequency of occurrence of integrase gene is comparable to that reported by others [16]. Odumosu et al (2013) [17] who reported the presence of class 1 integron in 31 (57%) multidrug resistant clinical isolates of *P.aeruginosa* but none of them carried class 2 and class 3 integrons. Cicek *et al* (2013) [18] screened for Class1 and 2 integrons but amplified class 1 integrons only in 10/205 (4.87%) isolates. Class 1 integrons are the most widely disseminated type in pathogens of human and animal origins [19] and have also been found in soil and aquatic ecosystems [20].

We observed the presence of bla_{VIM-2} gene and int1 gene in one isolate, Pa138 only. Several studies across the world describe the prevalence of class 1 integrons among metallo-β-lacatamase positive isolates of *P. aeruginosa*. In a study from China, 45.3% of the bla_{VIM} positive isolates carried integrons

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[21]. Lepšanović et al (2012) [22] found class 1 integrons in all the bla_{VIM} positive isolates of *P. aeruginosa* in Belgrade (Serbia). Kouda et al from Japan (2009) [23] reported the prevalence of class1 integrons in all the bla_{IMP-1} and bla_{VIM-2} positive isolates.

The integrons carry genes resistant to antibiotics such as sulphonamide (*sul1*), quaternary ammonium compounds (*qacEΔ1*). Integron class 1 is characterized by two conserved regions, the 5' conserved segment (5'-CS), which includes the integrase gene (*int1*), the adjacent recombination site (*attI1*) and the promoter (Pc), and the 3' conserved segment (3'-CS), which contains the *qacEΔ1* gene and *sul1*and the orf5 [24]. Sul1 codes for the enzyme dihydropteroate synthases which are responsible for resistance to sulphonamides. This gene is mostly found linked to other resistance genes in class 1 integrons including carbapenemases [25]. In order to see the association of *sul1* gene, we achieved amplification in 14 isolates by PCR. This gene was found linked to integron class 1 in case of nine isolates in the present investigations. This finding suggests that either class 1 integrons in our isolates have lost the *sul1* gene region or that this gene is carried on another genetic context. Similar findings have been reported by other workers also [26]. The studies carried out with respect to the presence of integron 1 and *sul1* reflect that they co-exist in a larger proportion. High prevalence of *sul1* gene (95%) was observed among Gram negative bacilli in Thailand [27]. Similarly Gundogdu et al (2011) [26] observed a high prevalence of 72% of *sul1* gene among *E. coli* isolates recovered from patients having UTI. Of these 55.1% were positive for class 1 integrase. Antunes et al (2005) [28] reported a high prevalence of *sul1* gene (76%) and also had class 1 integrons (77%) in *Salmonella enterica* strains in Portugal. These workers reported 96.7% isolates carrying class 1 integron and *sul1* gene. In another study from Mangalore (India), all the 18 cotrimoxazole (sulphonamide) resistant strains of *Salmonella spp*. isolated from human, poultry and seafood sources tested positive for *sul1* gene [29]. Shahid et al (2014) [30] observed 40.74% and 58.39% of environmental and clinical isolates of Gram negative bacteria respectively, carrying sul1 gene. Ho et al (2009) [31] also found a co-relation of integron class 1 and sulphonamide resistance gene (*sul1*) as a conserved segment of an integron class 1 component.

Conclusion

It may be concluded from the present study that multidrug resistant MBL and integron positive strains are prevalent in the state of Himachal Pradesh. We report the occurrence of class 1 integron and VIM-2 gene in this region. The nucleotide sequence analysis of the amplicons of these genes revealed 99-100% homology among isolates of *P. aeruginosa* as well as other Gram- negative bacteria. Further studies are however required in order to understand the epidemiology of *P. aeruginosa* strains circulating in the community in the state of Himachal Pradesh which would ultimately be helpful in the proper management of infections due to *P. aeruginosa* in this region.

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