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MOLECULAR CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* ISOLATES RECOVERED FROM HUMAN PATIENTS IN HIMACHAL PRADESH (INDIA) FOR SELECTIVE GENES: EXTENDED SPECTRUM β-LACTAMASE (ESBL), AMPICILLIN CLASS C (AMPC) AND METALLO β-LACTAMASE (MBL) GENES

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Keywords:

ESBL, AmpC, MBL, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{PDC}, bla_{NDM} , *P. aeruginosa*

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ABSTRACT: The present study highlights the occurrence of β-lactamase (*bla*) genes belonging to different classes: extended spectrum β-lactamase genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{PER}), metallo β-lactamase genes (bla_{NDM} and bla_{GIM} and *Ampicillin* class C genes (bla_{PDC} *and* bla_{CMY}) *in P. aeruginosa* isolates by PCR amplification. The isolates were recovered from human patients of Indira Gandhi Medical College & Hospital at Shimla in the state of Himachal Pradesh (India). We have previously detected different β-lactamases in 180 confirmed isolates of *P. aeruginosa* by phenotypic methods in our laboratory. Such isolates have been characterized in the present study for their genotypes in respect of the specified genes. Of the 56 phenotypically ESBL positive isolates, the ESBL genes (bla_{TEM}, bla_{SHV} and bla_{CTX-M}) could be amplified in 14 (25%), 1 (1.78%) and 6 (10.71%) isolates respectively. Of these, three isolates had both bla_{TEM} & bla_{CTX-M} genes. Of the 29 phenotypically MBL positive isolates, the bla_{NDM} could be amplified in 11 (37.93%) isolates only. However, no amplification was achieved in case of bla_{GIM} gene. AmpC-type gene derived from *P. aeruginosa* (bla_{PDC}) was successfully amplified in 11 out of 52 phenotypically AmpC positive isolates i.e. (21.15%). However, the amplification was not achieved in case of bla_{CMY} gene. The data reflect the occurrence of different classes of β-lactamase genes and their co-occurrence in some isolates. Based on nucleotide sequence homologies of amplicons of different genes, the NDM variants were identified as -1 and -7, TEM-1, SHV-12, CTX-M-15 in this geographic region, while the amplicon of bla_{PDC} showed homology to different bla_{PDC} variants.

INTRODUCTION: *Pseudomonas aeruginosa* is amongst the leading cause of nosocomial infections responsible for 10-15% of such infections worldwide. [1] This organism is intrinsically resistant to several antimicrobial agents.

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Besides, *P. aeruginosa* produces enzymes commonly known as β-lactamases which catalyze the hydrolysis of amide bond of β-lactam ring present in the β-lactam antibiotics, consequently helping the spread of β-lactam resistant strains. More than 500 different β-lactamases have been reported so far. [2]

The genes encoding these enzymes were originally located on the bacterial chromosome but may also be present on the plasmid or other accessory genome.[3-4] The resistance to β-lactam antibiotics is

increasing worldwide in Gram negative bacilli through the production of extended spectrum β-lactamase (ESBL) and Amp C β-lactamase enzymes. [5] Extended spectrum β-lactamases are capable of hydrolyzing oxyimino cephalosporins. These enzymes belong to class A of Ambler molecular classification scheme while AmpC β-lactamases belong to class C. Predominant ESBL genes that have been reported in *P. aeruginosa* include: TEM (Temoneira), SHV (sulfhydryl variable) and CTX-M (cefotaximase), PER (Pseudomonas extended spectrum beta-lactamase), VEB (Vietnamese extended spectrum beta-lactamase) and GES (Guiana extended spectrum) types from different parts of the globe. [6]

β-lactamase inhibitors such as clavulanic acid, tazobactam, sulbactam inhibit ESBLs. However, AmpC producers show resistance to clavulanic acid. AmpC producing organisms may act as a hidden reservoir for ESBL producing isolates and their high level expression may mask the recognition of ESBL producers.[7] AmpC-type β-lactamases may be carried on chromosome or plasmids of bacterial species. Furthermore, these enzymes are inducible and constitutively expressed in low quantities. On the basis of their antibiotic resistance profiles, AmpC-type genes are: CMY (cephamycins), FOX (cefoxitin), MOX (moxalactam) or LAT (latamoxef). The nomenculature of AmpC-types is based on i. the bacterial spp. from which the enzyme cephalosporinase is derived e.g. PDC (Pseudomonas derived cephalosporinase), ADC (Acinetobacter derived cephalosporinase) ii. name of the hospital from where the isolate was recovered e.g. MIR (Miriam hospital in Providence), DHA (Dhahran hospital in Saudi Arabia) and iii. name of the patient e.g. BIL (Bilal).

The carbapenems, β-lactams and β-lactamase inhibitor combinations such as piperacillin plus tazobactam are the drugs active against ESBL and AmpC producing *P. aeruginosa* isolates.[5] However, resistance to these drugs has also been increasing worldwide with the emergence of metallo β-lactamases. [8] These enzymes can hydrolyze all classes of β-lactam drugs with the exception of monobactams such as aztreonam and can resist neutralization by β-lactamase inhibitors. [9] Resistance to carbapenems is predominantly mediated by metallo β-lactamases. These enzymes belong to class B of Ambler classification system. The production of these enzymes not only limits the therapeutic options but is a matter of serious concern for infection control management.[10] Early detection of ESBL, AmpC and MBL producing organisms is crucial to establish appropriate antimicrobial therapy and to prevent their dissemination.[11]

On the basis of amino acid sequence homology, MBL types have been recognized as: IMP (Imipenemase), VIM (Verona integron encoded metallo β-lactamase), NDM (New Delhi metallo β-lactamase), SPM (Sao Paulo metallo β-lactamase), GIM (German imipenemase) and SIM (Seoul imipenemase).[12] The present study attempts to pinpoint the occurrence of selective genotypes of β-lactamase (ESBL, AmpC and MBL) genes in this geographic region of India.

MATERIALS AND METHODS:

Ethical statement:

The study was conducted at Department of Microbiology, Shoolini University, Solan (H.P), India. The research projects were cleared by the Institutional Ethics Committee (IEC) of the University through letter no. SUIEC/13/29 dated 10/4/2013 and letter no. SUIEC/13/30 dated 10/4/2013.

Bacterial isolates:

The present study is an extension of our earlier investigation, in which *P. aeruginosa* isolates were recovered from human patients at Indira Gandhi Medical College, Shimla, H.P. (India) in which 49/180 (27.22%) isolates were found resistant to multiple drugs (MDR) by *in vitro* antibiotic cultural sensitivity assay, 52/180 (28.89%) *P. aeruginosa* isolates were positive for AmpC production and 29/180 (16.11%) for MBL production and 56/180 (31.11%) were recorded positive for ESBL production by phenotypic assays. [32, 15, 14] The phenotypically positive isolates were further studied for the existence of selective ESBL (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{PER}) MBL (bla_{NDM}, bla_{GIM}) and AmpC (bla_{PDC}, bla_{CMY}) genes.

Extraction of genomic DNA:

The extraction of genomic DNA of each isolate was carried out by using Invitrogen genomic DNA isolation kit (Catalogue. no. K1820-01) according to the procedure described by the manufacturers.

Primer designing:

The primers were designed by using reference sequences of the genes in the GenBank database with the help of Primer3Plus tool. The detail of primer pairs used in the PCR assays for amplifying the selective β-lactamase genes are given in **Table 1**.

TABLE 1: DETAIL OF THE PRIMERS USED IN THE STUDY

Sr. No.	Gene	Primer sequence (5'-3')	Product size	Reference
1.	bla_{TEM}	**F:** TTCTGCTATGTGGTGCGGTA **R:** TTATCCGCCTCCATCCAGTC	424bp	Self designed
2.	bla_{SHV}	**F:** AAACGGAACTGAATGAGGCG **R:** ATACAATCAGGTGGCCACGT	530bp	Self designed
3.	bla_{PER}	**F:** CAACCTGCGCAATGATAGCT **R:** AAAGGTGCTGCAGAGATCCT	309bp	Self designed
4.	bla_{CTX-M}	**F:** GACGATGTCACTGGCTGAGC **R:** AGCCGCCGACGCTAATACA	499bp	Self designed
5.	bla_{PDC}	**F:** AGAAGGACCAGGCACAGATC **R:** CTCGGCATTGGGATAGTTGC	671bp	Self designed
6.	bla_{CMY}	**F:** CTGCACTTAGCCACCTATAC **R:** CCGTTTTATGCACCCATGAG	650bp	Self designed
7.	bla_{NDM}	**F:** CAAATGGAAACTGGCGACCA **R:** GCCTTGCTGTCCTTGATCAG	475bp	Self designed
8.	bla_{GIM}	**F:** CTGCACTTAGCCACCTATAC **R:** CCGTTTTATGCACCCATGAG	572bp	Self designed

PCR amplification for β-lactamase genes:

The extracted DNAs were subjected to PCR amplification of bla_{TEM}, bla_{SHV}, bla_{PER}, bla_{CTX-M} genes of 56 phenotypically positive isolates; bla_{PDC}, bla_{CMY} genes of 52 phenotypically positive isolates; bla_{NDM} and bla_{GIM} genes of 29 phenotypically positive isolates. In house strains of *P. aeruginosa* PA38, PaIg27, PA1013, E412, PA91, PA126, *K. pneumoniae* ATCC 700603 used as positive controls and *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 were used as negative controls in PCR assays. The reaction conditions of which are shown in **Tables 2** and **3**.

TABLE 2: AMPLIFICATION OF BLA_{TEM}, BLA_{SHV}, BLA_{PER} AND BLA_{CTX-M} GENES OF SELECTIVE *P. AERUGINOSA* ISOLATES BY PCR

Sr No.	Gene	Reaction contents		Reaction conditions		
		Contents	Final volume (25 µl)	PCR steps	Temperature and Time	Cycles
1.	bla_{TEM}	Target DNA	2µl	Intial denaturation	95°C- 3 mins	
		10X PCR buffer	2.5µl	Denaturation	95°C- 30 secs	
		$MgCl_2$ (25mM)	0.8µl	Annealing	56°C-30secs	
		DNTPs (10mM)	2.5 µl ×4	Extention	72°C- 45secs	35
		Primers (20 pmol)	1+1µl	Final extention	72°C-10 mins	
		Taq polymerase	0.3µl	Holding	4°C- ∞	
		PCR water	7.4µl			
2.	bla_{SHV}	Target DNA	2	Intial denaturation	95°C - 3mins	
		10X PCR buffer	2.5	Denaturation	95°C – 30 secs	
		$MgCl_2$ (25mM)	0.8	Annealing	55.5°C- 30secs	35
		DNTPs (10mM)	2×4	Extention	72°C- 45 sec	
		Primers (20 pmol)	1+1	Final extention	72°C- 10 mins	
		Taq polymerase	0.2	Holding	4°C- ∞	
		PCR water	9.5			
3.	bla_{PER}	Target DNA	2	Intial denaturation	95°C- 3mins	
		10X PCR buffer	2.5	Denaturation	95°C-30 secs	
		$MgCl_2$ (25mM)	0.8	Annealing	56°C-30 secs	

		DNTPs (10mM)	2×4	Extention	72°C-40 secs	35
		Primers (20 pmol)	1+1	Final extention	72°C-10 mins	
		Taq polymerase	0.3	Holding	4°C-∞	
		PCR water	9.4			
4.	***bla*$_{CTX-M}$**	Target DNA	2	Intial denaturation	95°C- 3 mins	
		10X PCR buffer	2.5	Denaturation	95°C- 30 secs	
		$MgCl_2$ (25mM)	0.75	Annealing	57°C- 30 secs	
		DNTPs (10mM)	2.5×4	Extention	72°C-1min30 secs	35
		Primers (20 pmol)	1+1	Final extention	72°C- 10 mins	
		Taq polymerase	0.3	Holding	4°C- ∞	
		PCR water	7.45			

TABLE 3: AMPLIFICATION OF *BLA*$_{NDM}$, *BLA*$_{GIM}$, *BLA*$_{PDC}$ AND *BLA*$_{CMY}$ GENES OF SELECTIVE *P. AERUGINOSA* ISOLATES BY PCR

Sr No.	Gene	Reaction contents		Reaction conditions		
		Contents	Final volume (25µl)	PCR steps	Temperature & Time	Cycles
1.	***bla*$_{NDM}$**	Target DNA	2	Initial denaturation	95°C- 3mins	35
		10X PCR buffer	2.5	Denaturation	95°C- 30sec	
		$MgCl_2$ (25mM)	0.75	Annealing	56.5°C- 30sec	
		DNTPs (10mM)	2.5 each	Extension	72°C- 45sec	
		Primers (20 pmol)	1 each	Final extension	72°C- 10mins	
		Taq polymerase	0.3	Holding	4°C-∞	
		PCR water	7.45			
2.	***bla*$_{GIM}$**	Target DNA	2	Initial denaturation	95°C- 3 mins	35
		10X PCR buffer	2.5	Denaturation	95°C- 30 sec	
		$MgCl_2$ (25mM)	0.75	Annealing	58°C- 30 sec	
		DNTPs (10mM)	2.5 each	Extension	72°C- 40 sec	
		Primers (20 pmol)	1 each	Final extension	72°C- 10 mins	
		Taq polymerase	0.3	Holding	4°C-∞	
		PCR water	7.45			
3.	***bla*$_{PDC}$**	Target DNA	2	Initial denaturation	95°C- 3 min's	35
		10X PCR buffer	2.5	Denaturation	95°C- 30 sec's	
		$MgCl_2$ (25mM)	0.8	Annealing	60°C- 30 sec's	
		DNTPs (10mM)	2.5	Extension	72°C- 45 sec's	
		Primers (20 pmol)	1 each	Final extension	72°C-10 min's	
		Taq polymerase	0.25	Holding	4°C-∞	
		PCR water	7.45			
4.	***bla*$_{CMY}$**	Target DNA	2	Initial denaturation	95°C- 3 min	35
		10X PCR buffer	2.5	Denaturation	95°C-30 sec	
		$MgCl_2$ (25mM)	0.75	Annealing	55°C-30 sec	
		DNTPs (10mM)	2.5 each	Extension	72°C- 1 min 20 sec	
		Primers (20 pmol)	1 each	Final extension	72°C- 10 min	
		Taq polymerase	0.3	Holding	4°C-∞	
		PCR water	7.45			

Nucleotide sequencing of the amplicons:

Nucleotide sequencing of the amplicons of different genes of selective *P. aeruginosa* isolates was done by Molecular Diagnostics and Research Laboratories (MDRL), a commercial facility at Chandigarh, using Sanger dideoxynucleotide sequencing method. Amplicons of three *P. aeruginosa* isolates were sequenced for each gene: *bla*$_{TEM}$ (PA38, PaIg53, PaIg76); *bla*$_{CTX-M}$ (PaIg27, PaIg29, PA137); *bla*$_{PDC}$ (PA91, PA41, PaIg20), *bla*$_{NDM}$ (PA126, PaIg20, PaIg53). However, single isolate (PaIg48) was sequenced for *bla*$_{SHV}$.

Determination of nucleotide sequence homologies:

The nucleotide sequences obtained were subjected to $BLAST_n$ analysis to recognize the percent homology with the published sequences of National Center for Biotechnology Information (NCBI). Multiple sequence alignment was done to verify the variations among isolates and with their respective reference sequences by using CLUSTAL OMEGA software tool.

Submission of sequences of the amplicons to the NCBI:

The nucleotide sequences were submitted to Gen Bank (www.ncbi.nlm.gov/genbank/) using Bank It submission tool to obtain accession numbers.

RESULTS:

PCR amplification of *bla_{TEM}*, *bla_{SHV}*, *bla_{PER}* and *bla_{CTX-M}* (ESBL genes): The genomic DNAs of 56 isolates of *P. aeruginosa* were used as templates for PCR amplification of the ESBL genes (*bla_{TEM}*, *bla_{SHV}* and *bla_{CTX-M}*). The band sizes of the amplicons of these genes were recorded as 424bp, 530bp and 499bp respectively **(Fig.1, Fig.2** and **Fig.3)**. Further, amplification in respect of above *bla* genes was observed in 14/56 (25%), 1/56 (1.78%) and 6/56 (10.71%) isolates respectively **(Table 4)**. However, the amplification of *bla_{PER}* gene of the isolates was not achieved in the PCR assays.

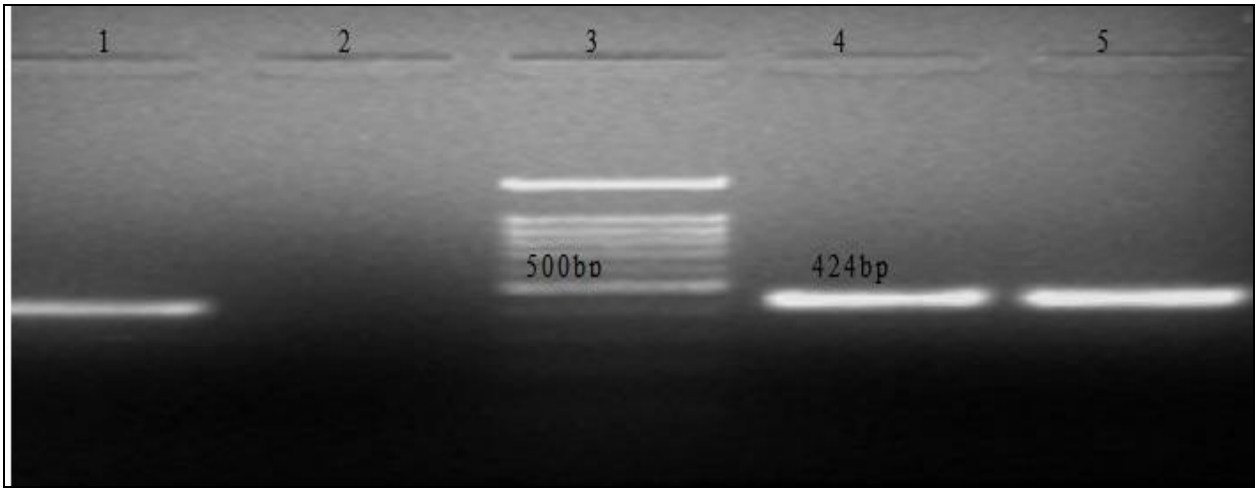

FIG. 1: AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS OF *bla_{TEM}* GENE AMPLICONS OF ISOLATE NO'S PaIg53 AND PaIg76 (lane 4 and 5) HAVING EXPECTED BAND SIZE OF 424bp. LANE 3 CONTAINS 100bp DNA MARKER. LANE 1 WAS LOADED WITH POSITIVE CONTROL (IN HOUSE STRAIN, PA38) AND LANE 2 WAS LOADED WITH NEGATIVE CONTROL (*E. COLI* ATCC 25922)

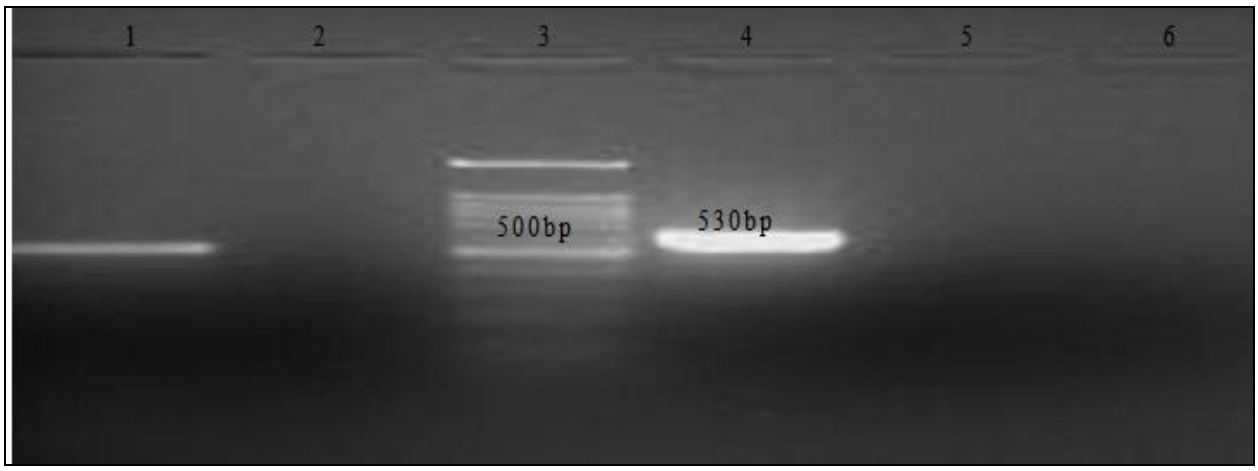

FIG. 2: AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCT OF *bla_{SHV}* GENE AMPLICON OF ISOLATE NO. PAIG48 (LANE 4). A BAND OF ~ 530bp IS VISIBLE IN THE LANE 4. AMPLIFICATION WAS, HOWEVER NOT OBSERVED IN LANE 5 (ISOLATE PaIg47) AND LANE 6 (ISOLATE PA51). LANE 3 WAS LOADED WITH 100bp DNA MARKER. LANE 1 CONTAINS AMPLICON OF POSITIVE CONTROL (*K. PNEUMONIAE* ATCC 700603) AND LANE 2 WAS LOADED WITH NEGATIVE CONTROL (*E. COLI* ATCC 25922)

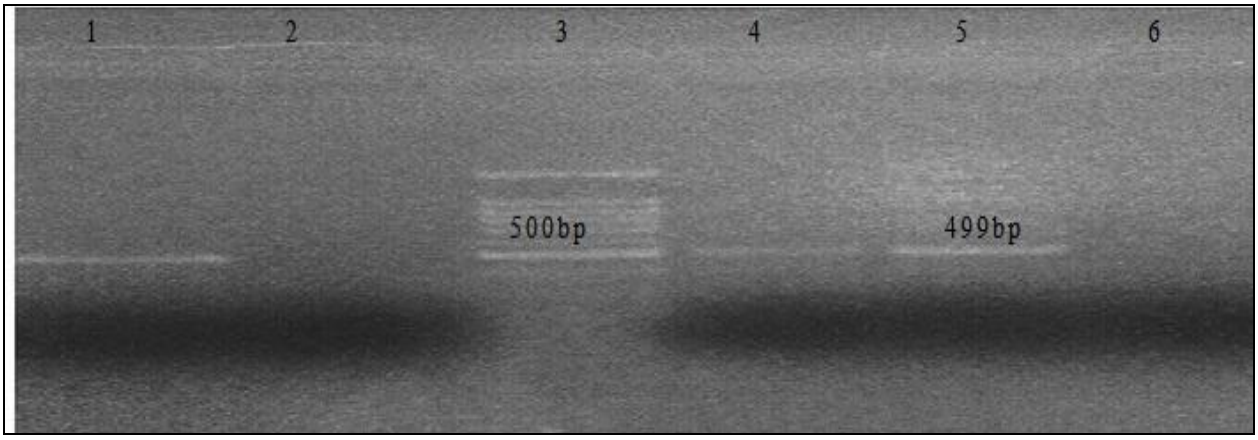

FIG. 3: AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS OF *bla*$_{CTX\text{-}M}$ GENE AMPLICONS OF ISOLATES PA137 AND PaIg29 (lane 4 and 5) HAVING BAND OF EXPECTED SIZE OF 499bp. AMPLIFICATION WAS NOT OBSERVED IN CASE OF ISOLATE NO. PA122 (LANE 6), LANE 3 WAS LOADED WITH 100bp DNA MARKER. AMPLICON OF POSITIVE CONTROL (IN HOUSE STRAIN, PaIg27) WAS LOADED IN LANE 1 AND NEGATIVE CONTROL (*E. COLI* ATCC 25922) (LANE 2)

PCR amplification of *bla*$_{PDC}$ and *bla*$_{CMY}$ (AmpC genes): The expected band size of 671bp was observed on agarose gel electrophoresis of PCR products of *bla*$_{PDC}$ gene amplicons **(Fig.4)**. The amplification was achieved in 11/52 (21.15%) isolates **(Table 4)**. However, no amplification was observed in case of *bla*$_{CMY}$ gene.

PCR amplification of *bla*$_{NDM}$ and *bla*$_{GIM}$ (MBL genes): A band of expected size of 475 base pairs was observed on agarose gel electrophoresis of the *bla*$_{NDM}$ gene amplicons **(Fig.5)** The amplification of *bla*$_{NDM}$ gene was observed in 11/29 (37.93%) isolates **(Table 4)**. However, the amplification of *bla*$_{GIM}$ gene was not observed among the isolates tested.

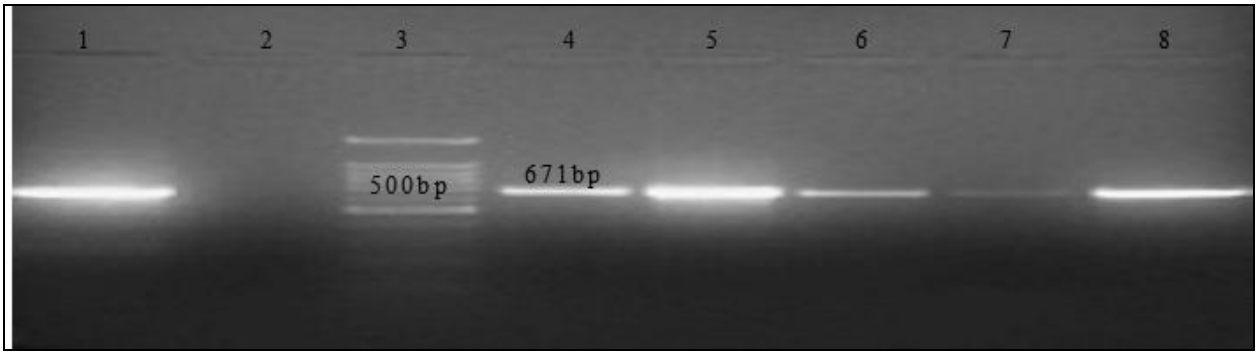

FIG. 4: AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS OF *bla*$_{PDC}$ GENE AMPLICONS OF ISOLATES PA91, PaIg72, PA108, PA101 AND PA126 (lanes 4 to 8) HAVING EXPECTED BAND SIZE OF 671bp. LANE 1 WAS LOADED WITH POSITIVE CONTROL (*K. PNEUMONIAE* ATCC 700603) AND NEGATIVE CONTROL (*P. AERUGINOSA* ATCC 27853) (LANE 2). LANE 3 WAS LOADED WITH100bp DNA MARKER

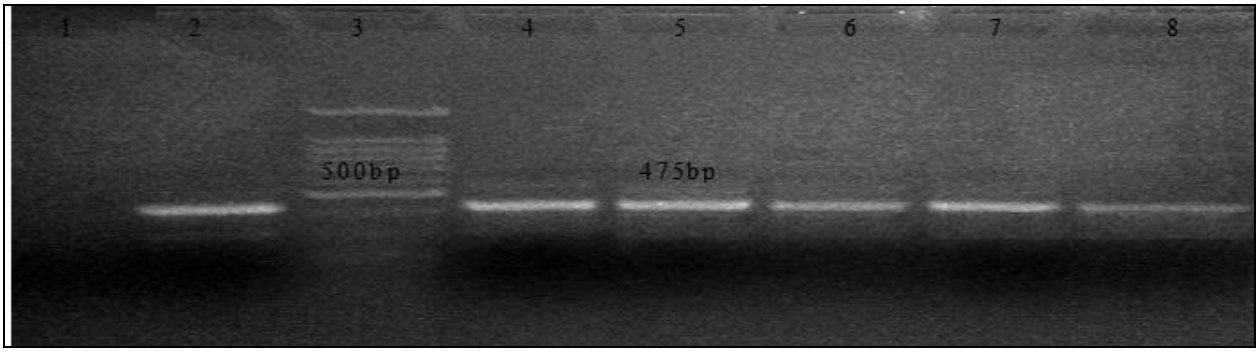

FIG.5: AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS OF *bla*$_{NDM}$ GENE AMPLICONS OF ISOLATES PaIg20, PaIg53, PA41, PaIg22 and PaIg17 (lanes 4 to 8) HAVING EXPECTED BAND SIZE OF 475bp. LANE 3 WAS LOADED WITH 100bp DNA MARKER. AMPLICON OF POSITIVE CONTROL (IN HOUSE STRAIN, PA126) WAS LOADED IN LANE 2 AND NEGATIVE CONTROL (*E. coli* ATCC 25922) IN LANE 1

Co-occurrence of the ESBL, MBL and AmpC β-lactamase genes:

The co-occurrence of ESBL (bla_{TEM}) and MBL (bla_{NDM}) gene was observed in a single isolate (PA18) only out of eight positive by phenotypic tests **(Table 4)**, while co-existence of AmpC (bla_{PDC}) and MBL (bla_{NDM}) gene was observed in four isolates (PaIg70, PaIg20, PA126 and PA41) out of fourteen phenotypically positive isolates **(Table 4)**. Two isolates (PA141 and PaIg53) had all the three β-lactamase classes: ESBL (bla_{TEM}), MBL (bla_{NDM}) and AmpC (bla_{PDC}) out of five phenotypically positive isolates **(Table 4)**. However, devoid of MBL genotype, the co-occurrence of ESBL and AmpC genotypes was not observed in any of the 27 phenotypically positive isolates. The details of occurrence of *bla* genes alone or in combination in *P. aeruginosa* isolates detected by PCR assays are presented in **Table 5**.

TABLE 4: THE DETAILS OF FREQUENCIES OF DIFFERENT *BLA* GENE CLASSES AMONG *P. AERUGINOSA* ISOLATES

Total no. of isolates examined	β-lactamase class	Phenotypically positive isolates	Genotypically positive isolates
180	ESBL	56/180 (31.11%)	21/56 (37.5%)
	AmpC	52/180 (28.89%)	11/52 (21.15%)
	MBL	29/180 (16.11%)	11/29 (37.93%)
	ESBL+AmpC	27/180 (15%)	0/27 (0%)
	MBL+AmpC	14/180 (7.78%)	4/14 (28.58%)
	ESBL+MBL	8/180 (4.44%)	1/8 (12.5%)
	ESBL+MBL+AmpC	5/180 (2.78%)	2/5 (40%)

TABLE 5: THE DETAILS OF OCCURRENCE OF *BLA* GENES ALONE OR IN COMBINATION IN *P. AERUGINOSA* ISOLATES

β-lactamase class	Isolate	Detection of genes by PCR assays	No. of isolates having single gene or combination of genes
ESBL gene type alone or in combination	PA35	bla_{TEM}	8
	PaIg47	bla_{TEM}	
	PaIg08	bla_{TEM}	
	PA51	bla_{TEM}	
	PA38	bla_{TEM}	
	PaIg12	bla_{TEM}	
	PA22	bla_{TEM}	
	PaIg76	bla_{TEM}	
	PaIg27	bla_{TEM}+bla_{CTX-M}	3
	PA31	bla_{TEM}+bla_{CTX-M}	
	PaIg50	bla_{TEM}+bla_{CTX-M}	
	PaIg48	bla_{SHV}	1
	PA137	bla_{CTX-M}	3
	PA147	bla_{CTX-M}	
	PaIg29	bla_{CTX-M}	
MBL	PA87	bla_{NDM}	4
	PaIg17	bla_{NDM}	
	PaIg19	bla_{NDM}	
	PaIg22	bla_{NDM}	
AmpC	PaIg72	bla_{PDC}	5
	PaIg76	bla_{PDC}	
	PA108	bla_{PDC}	
	PA101	bla_{PDC}	
	PA91	bla_{PDC}	
ESBL+MBL	PA18	bla_{TEM} + bla_{NDM}	1
MBL+AmpC	PaIg70	bla_{NDM} +bla_{PDC}	4
	PaIg20	bla_{NDM} +bla_{PDC}	
	PA126	bla_{NDM} +bla_{PDC}	
	PA41	bla_{NDM} +bla_{PDC}	
MBL+AmpC+ ESBL	PA141	bla_{NDM} +bla_{PDC}+ bla_{TEM}	2
	PaIg53	bla_{NDM} +bla_{PDC}+ bla_{TEM}	

Nucleotide sequencing of the amplicons of different *bla* genes:

The nucleotide sequencing of the amplicons of three isolates for each gene was done: bla_{TEM} (PA38, PaIg53, PaIg76); bla_{CTX-M} (PaIg27, PaIg29, PA137); bla_{PDC} (PA91, PA41, PaIg20), bla_{NDM} (PA126, PaIg20, PaIg53). In addition, bla_{SHV} gene amplicon of a single isolate (PaIg48) detected positive in PCR assays was also sequenced. The sequence homologies of these nucleotides were determined by $BLAST_n$ analysis of published NCBI sequences:

$BLAST_n$ analysis of bla_{TEM} gene amplicons of isolates PaIg53, PaIg76 and PA38.

Bla_{TEM} gene amplicon of isolate PaIg53 showed 99% homology with $bla_{TEM\text{-}1}$ gene variant of standard NCBI sequences of *Escherichia coli* strain RIGLD-1B9-F1 (accession no. KP308219.1) and *Klebsiella pneumoniae* strain T3 (KR303752.1). Isolate PaIg76 revealed 99% homology with $bla_{TEM\text{-}1}$ gene variant of standard NCBI sequences of uncultured soil bacterium clone M5 (EF514086.1), *E. coli* strain RIGLD-1B9-F1 (KP308219.1) and *K. pneumoniae* strain T3 (KR303752.1). The exact variant of bla_{TEM} gene was not clearly established in case of isolate PA38 as it showed homology with different variants of bla_{TEM} gene of different standard NCBI strains (accession nos. KP853090.1, KP853089.1, KP853092.1 and KP853091.1). The nucleotide sequence homologies of the gene amplicons of three isolates sequenced ranged from 97.22% to 99.74% among themselves.

$BLAST_n$ analysis of bla_{SHV} gene amplicon of the isolate PaIg48.

Bla_{SHV} gene amplicon of isolate PaIg48 showed 100% homology with $bla_{SHV\text{-}12}$ gene variant of standard NCBI sequences of Acinetobacter spp. SVU/SVIMS1 (KJ083256.1), *E. coli* strain H59L (KM011336.1), *Citrobacter freundii* (AY940490.1) and K. pneumoniae (KF585138.1).

BLASTn analysis of bla_{CTX-M} gene amplicons of the isolates PaIg27, PaIg29 and PA137.

Amplicon of the bla_{CTX-M} gene of isolate PaIg27 showed 98% homology with $bla_{CTX-M-15}$ gene variant of standard NCBI sequences of *E. coli* strain V512 (LC095574.1), *E. coli* strain V508 (LC095573.1), *E. coli* strain V501 (LC095572.1) and *E. coli* strain V496 (LC095571.1). Bla_{CTX-M} gene amplicon of isolate PaIg29 showed 99% sequence homology with $bla_{CTX-M-15}$ gene variant of standard NCBI sequences of *E. coli* strain NK-73 (KP849465.1), *K. pneumoniae* strain 628 (KP987217.1), *E. coli* strain NK-76 (KP849464.1) and *K. pneumonia*e strain KP80 (KP698226.1) and isolate PA137 revealed 99% homology with $bla_{CTX\text{-}M\text{-}15}$ gene variant of standard NCBI sequences of *K. pneumoniae* strain 628 (KP987217.1), *E. coli* strain NK-73 (KP849465.1), *E. coli* strain NK-76 (KP849464.1) and *K. pneumonia*e strain KP30 (KP698224.1). The amplicons of this gene showed 86.67% to 98.86% homology among themselves on alignment.

$BLAST_n$ analysis of bla_{PDC} gene amplicons of the isolates PA91, PA41 and PaIg20.

Bla_{PDC} gene amplicon of isolate PA91 showed 99% homology with different bla_{PDC} gene variants of standard *P. aeruginosa* strains of NCBI database (KJ949063.1, KR057762.1, KJ949046.1 and AB211126.1). Isolate PA41 showed 99% sequence homology with different bla_{PDC} gene variants of standard NCBI strains (KJ949082.1, KJ949060.1, KJ949070.1 and KJ949048.1) and isolate PaIg20 also showed 99% homology with different bla_{PDC} gene variants of standard NCBI strains (AP014839.2, FR822745.1, KJ949055.1 and AY083594.1).

The nucleotide sequence homologies of the gene amplicons of three isolates sequenced ranged from 97.04% to 97.81% among themselves. However, distinct conserved sequences were observed among the three isolates (PA91, PA41, PaIg20) on alignment of nucleotide sequences of amplicons of bla_{PDC} gene amplicons.

BLASTn analysis of bla_{NDM} gene amplicons of the isolates PA126, PaIg20 and PaIg53.

Bla_{NDM} gene amplicon isolate PA126 showed 99% homology with $bla_{NDM\text{-}1}$ variant of different standard NCBI sequences of *E. coli* strains V308 (LC095548.1), V266 (LC095524.1) and

Pseudomonas spp. strains NF81 (KP772171.1), NF117 (KP772196.1). Isolate PaIg20 showed 99% homology with bla_{NDM-7} variant of different standard NCBI *E. coli* strains V46 (LC095463.1), V5 (LC095457.1), V4 (LC095455.1) and V2 (LC095452.1). The exact variant of bla_{NDM} gene of isolate PaIg53 was not clearly identified as it showed homology with different standard NCBI strains of *E. coli* NF92 (KP772213.1), strain 15 (JQ348841.1), strain CR53 (KP826711.1) and *Pseudomonas* spp. strain NF91 (KP772212.1) possessing different variants of bla_{NDM} gene. The nucleotide sequence homologies of the gene amplicons of three isolates sequenced ranged from 86% to 98.77% among themselves. However, distinct conserved sequences were observed among the three isolates (PA126, PaIg20, PaIg53) on alignment of nucleotide sequences of amplicons of bla_{NDM} genes.

Nucleotide sequence accession numbers:

The nucleotide sequence data in respect of different β-lactamase genes submitted to the National Center for Biotechnology Information (NCBI) were assigned different accession numbers: KU139122 (PA38), KU139123 (PaIg53), KU139119 (PaIg76) for bla_{TEM}, KU058668 (PaIg48) for bla_{SHV}, KU139121 (PaIg27), KU139120 (PaIg29), KU139118 (PA137) for bla_{CTX-M}, KT989611 (PA91), KT989612 (PA41), KT989613 (PaIg20) for bla_{PDC} and KT966377 (PaIg20), KU058669 (PA126), KU058670 (PaIg53) for bla_{NDM}.

DISCUSSION:

The β-lactamases (AmpC, ESBLs and MBLs) pose major therapeutic challenge to the clinicians[13]. We have previously characterised 180 isolates of *P. aeruginosa* from Shimla region of India. The frequencies of different classes of β-lactamases of these isolates from this geographic region on phenotypic testing by phenotypic methods were recorded as: 31.11%, 28.89% and 16.11% respectively for ESBL, AmpC and MBL production.[14-15] Different researchers have reported different frequencies of the ESBL producers: Bandekar *et al*., (2011) observed 39.8% ESBL producers among Gram negative bacteria isolated from burn patients from Karnataka, India and Rafiee *et al*. (2014) reported 35.16% ESBL producers from Amritsar, India.[16, 13]

ESBL genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}) were amplified in 21 out of 56 phenotypically ESBL positive *P. aeruginosa* isolates i.e. 37.5%. We attempted the amplification of only four ESBL genes, therefore, the possibility of occurrence of other ESBL types in remaining ESBL positive *P. aeruginosa* isolates cannot be ruled out. The amplification of bla_{TEM}, bla_{SHV} and bla_{CTX-M} was achieved in 14 (25%), 1 (1.7%) and 6 (10.71%) isolates respectively among 56 *P. aeruginosa* isolates. We report the prevalence of bla_{TEM-1}, bla_{SHV-12} and $bla_{CTX-M-15}$ variants in this geographic region of India on the basis of $BLAST_n$ analysis of nucleotide sequences of the amplicons.

However, bla_{PER} gene was not amplified in the PCR assays. Isolates (37.93%) were positive for bla_{NDM} gene by PCR amplification i.e. 11 out of 29 phenotypically MBL positive *P. aeruginosa* isolates. The occurrences of bla_{NDM-7} and bla_{NDM-1} variants were recorded in this geographic region of the country. The bla_{NDM-1} enzyme was named after New Delhi, India, as it was first detected in a *Klebsiella pneumoniae* isolate from a Swedish patient who fell ill with an antibiotic-resistant bacterial infection that he acquired in India in 2008.[17] It was later reported from several countries, India, Pakistan, the United Kingdom, the United States, Canada, and Japan etc. The frequency of bla_{NDM} (New Delhi metallo β-lactamase) was however, the highest i.e. 37.93%. Higher frequency (53.4%) of bla_{NDM-1} gene variant among Gram negative bacilli recovered at Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh has been observed.[18] This institute is located at a distance of about 120 Km from Shimla in northern part of India. Lower frequency of bla_{NDM} gene among *P. aeruginosa* isolates as compared to ours has been reported from Southern India.[19] Deshpande *et al*., (2010) from Mumbai (India) reported bla_{NDM} gene in *E. coli* isolates (n=9) among 24 carbapenem-resistant bacteria of *Enterobacteriaceae*.[20]

The antibiotic resistance genes are present on cassettes on class 1 integrons. In our laboratory, 40.74% isolates of *P. aeruginosa* had class 1 inetgron (*int1*) and 22.22% isolates had Verona imipenemase (bla_{VIM-2}) gene while 51.85% isolates had sulfonamide resistance (*sul1*) genes in a

previous study. [21] The present study is an extension in the direction of studying molecular epidemiology of *P. aeruginosa* isolates in this geographic region.

AmpC-type variants chromosomally derived from *P. aeruginosa* are also known as Pseudomonas derived cephalosporinase (*bla*$_{PDC}$) while Acinetobacter derived cephalosporinase is denoted by *bla*$_{ADC}$.[22] In the present study, 11 out of 52 isolates tested (21.15%) were found to have *bla*$_{PDC}$ gene by nucleotide sequencing of the amplicons of this gene. The exact variant of *bla*$_{PDC}$, however, could not be clearly established as it showed homology with various variants of *bla*$_{PDC}$ gene of different bacterial spp. Since we have amplified a fragment of 671bp only of this gene, the amplification of larger fragment might be useful for establishing the exact variant of this gene.

We, however, did not achieve amplification of *bla*$_{CMY}$ (cephamycin) gene in any of the isolates tested which suggests that another AmpC gene(s) or other beta-lactamase gene(s) may be present in these strains which might be responsible for resistance to cefepime (cephamycin) antibiotic in the phenotypic test. Alternatively, this resistance gene could be located on plasmid. Cefoxitin resistance in AmpC non-producers could be due to some other resistance mechanism such as, lack of permeation of porins or due to production of carbapenemases.[23]

Carbapenems are considered to be the drugs of last choice in the treatment of AmpC and ESBL producing *P. aeruginosa*. Extensive use of this drug during past few years has led to the increased levels of bacterial resistance mostly due to production of MBLs. In the present study, co-occurence of ESBL (*bla*$_{TEM-1}$) and MBL (*bla*$_{NDM}$) gene was detected in a single isolate. However, Bora *et al*. (2013) observed a high co-existence rate (57.14%) of ESBL genes (*bla*$_{TEM}$ and *bla*$_{CTX-M}$) with MBL (*bla*$_{NDM-1}$) gene in *E. coli* isolates from Assam, India.[24] In our study, co-occurence of AmpC (*bla*$_{PDC}$) and MBL (*bla*$_{NDM}$) genes was observed in four isolates (28.58%) **(Table 4)**. However, others did not observe co-existence of chromosomal AmpC and MBL genes in Gram negative bacilli. [25] Kumar *et al*. (2012) from Varanasi, India have reported 48.5% clinical isolates of *P. aeruginosa* that possessed AmpC + MBL genes.[26] Abd El-Baky *et al*., 2013 from Egypt reported a frequency (43.1%) of AmpC + MBL.[27] Interestingly, we recorded two isolates which possessed all the three classes of β- lactamase AmpC-type (*bla*$_{PDC}$), ESBL (*bla*$_{TEM}$) and MBL (*bla*$_{NDM}$) genes. The presence of multiple β-lactamases in a single isolate might contribute to the high degree of resistance which might lead to treatment failure and high mortality. Bora *et al*. (2013) from Assam, India reported co-existence of AmpC and ESBL genes (*bla*$_{AmpC}$ and *bla*$_{TEM}$, *bla*$_{CTX-M}$) with MBL gene (*bla*$_{NDM-1}$) in 21.43% *E. coli* isolates.[24]

In the present study, *bla*$_{TEM}$ was the most frequent ESBL gene followed by *bla*$_{NDM}$ (MBL) and *bla*$_{PDC}$ (AmpC). Highest frequency of occurrence of *bla*$_{TEM}$ gene was also reported by researchers from Iran.[28-29] The simultaneous occurrence of AmpC and ESBL genes was not observed in our study. However, 3.9% isolates produced AmpC and ESBL simultaneously from Amritsar, India.[13] Likewise, 3.3% isolates had AmpC and ESBL genes from Varanasi, India.[30] In another report from Varanasi, India co-production of AmpC and ESBL by 24.5% isolates of *P. aeruginosa* has been reported.[26] Similarly, the production of both ESBL (*bla*$_{CTX-M}$) and AmpC gene has been reported in 21.43% *E. coli* isolates from Assam, India.[24] Isolates that produce both an ESBL and a high level of AmpC are becoming more common problem. High level expression of AmpC can prevent recognition of ESBLs leading to false negative results.

In such cases, one should follow the methods to re-test the isolate which are unaffected by AmpC β-lactamases such as: incorporating an AmpC inhibitor such as cloxacillin in the culture medium; by including the AmpC inhibitor boronic acid in either MIC or disc tests; or by testing a cephalosporin (cefepime) which is not hydrolyzed by AmpC alone as well as a combination of clavulanate with other cephalosporins.[31]

CONCLUSION: In the present study, we have sequenced three isolates for amplicons of the genes (*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{PDC}$ and *bla*$_{NDM}$). Their results however, may not be complete reflection of

the occurrence of ESBL, MBL and AmpC genes in the *P. aeruginosa* isolates of this geographic region. Furthermore, the isolates can be studied further for other gene types of different β-lactamase classes. Such studies might prove useful for the clinicians for managing infections due to *P. aeruginosa* isolates in this region. However, the surveillance of the circulating strains of *P. aeruginosa* can be undertaken on regular and continuous basis.

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