

Original Article

Detection of Metallo-Beta-Lactamase Gene in Carbapenem Resistant *Pseudomonas Aeruginosa* Isolated From Lahore, Pakistan

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Abstract:

Pseudomonas aeruginosa is a widespread organism, caused severe nosocomial infection in human and associated with multiple drug resistance (MDR) **Objective:** The present study was carried out to observe current antimicrobial resistant pattern of *Pseudomonas aeruginosa* in Lahore and to detect the Metallo-beta-lactamase (MBL) gene in carbapenem resistant *Pseudomonas aeruginosa* **Methods:** By screening 360 samples total 123 *Pseudomonas aeruginosa* was identified by standard microbiology techniques such as microscopy and biochemical testing. The isolated *Pseudomonas aeruginosa* was evaluated for drug resistance by disc diffusion method and polymerase chain reaction (PCR) was used to identify the carbapenem resistance causing gene (bla-VIM and bla-IMP) **Results:** Following antibiotic resistant pattern was observed, Gentamycin (59.00%), Ceftazidime (58.7%), Ceftriaxone (58.00%), Cefotazime (57.0%) and Ciprofloxacin (55.00%). Resistance rates to carbapenem group of antibiotics is Doripenem (30.5%) Meropenem (31.0%) and Imipenem (28.0%). Out of 123 samples of *Pseudomonas aeruginosa*, 28 isolates were found resistant to carbapenem group of antibiotic which was supposed to be highly sensitive for this bacterium. Molecular based identification of resistance genes showed that bla-IMP gene was present in 32.1% (09) and bla-VIM was found positive in 17.8% (04) samples. Metallo-beta-lactamases producing genes (bla-VIM and bla-IMP), among carbapenem resistant *Pseudomonas aeruginosa* were detected in 28.1% of samples. If other carbapenem resistant gene were also included this number might be higher **Conclusions:** PCR based test should be included in routine laboratory examination for quick detection of the resistance causing genes.

Keywords: *Pseudomonas aeruginosa*, Metallo-beta-lactamase, carbapenem resistance**Introduction:**

Antibiotic was thought to be a magical bullet when first time discovered [1]. A simple single penicillin injection was considered enough for against life threatening bacterial infection [2]. Unfortunately as the time passes bacteria produce different genes and enzymes for resistant against antimicrobial agents [3,4,5,6] *Pseudomonas aeruginosa* is most important opportunistic pathogen which causes serious nosocomial infection, bacteremia, pneumonia,

urinary tract infections, meningitis, soft-tissue and skin infections [7,8].

Mechanisms that produce carbapenem resistance in *Pseudomonas aeruginosa* are due to presence of MBL resistance genes [9]. MBL producing *Pseudomonas aeruginosa* when causes infections are difficult to treat and increase health care cost. MBL is included in class B having divalent cations as cofactors which is required for optimal enzyme activity, and

repressed by metal ion chelator action. The detection of MBL is very significant for MDR treatment of *P. aeruginosa*. The most common gene MBLs includes the GIM, VIM, SIM, IMP, SPM and the newly identified NDM-1. In particular, bla VIM and bla-IMP has emerged as a leading MBL variant worldwide MBL producing enzyme gene bla-IMP in *Pseudomonas aeruginosa* was first time identified in Japan 1990 and bla-VIM was isolated from Italy in 1999 [10]. The present study was done to evaluate the current antimicrobial sensitivity pattern of *Pseudomonas aeruginosa* in Lahore and identify the presence of MBL producing gene, through PCR.

Methods:

Sample processing:

This study was done in the University of Lahore (Institute of Molecular Biology and Biotechnology). The present study was conducted during June 2015 to December 2015. Total 360, Urine (90 samples), Pus (90 samples), Sputum (90 samples) and blood (90 samples) samples were collected from different clinical labs. Urine samples were inoculated on CLED agar whereas pus and sputum samples were cultured on blood agar, MacConkey agar and were incubated overnight at 37 °C. Blood samples were preceded on Thioglycollate broth; incubate at 37 °C for 7 days then subculture the blood samples on blood and MacConkey agar.

Confirmation of organism:

After obtaining the pure culture growth the Gram staining was performed to identify the organism.

Biochemical tests were done by using Oxidase test and analytic profile index (API) kit.

Antimicrobial disc susceptibility test:

Disc diffusion method (Modified Kirby Baur) was used for antimicrobial susceptibility of all isolated *Pseudomonas aeruginosa* according to CLSI's guidelines. Following antibiotics were used, ceftazidime, cefotaxime, ceftriaxon, meropenem, imipenem, doripenem, gentamycin and ciprofloxacin.

Procedure of DNA isolation:

Carbapenem resistant 28 isolates of *Pseudomonas aeruginosa* were inoculated on Macconkey agar. Incubate overnight at 37 °C. Take 50µL distilled water in each eppendorf and transfer single isolated *Pseudomonas aeruginosa* colony through wire loop in it. Mix well by vortex mixer for 1 min. Heat them at 100 °C for 10 min. Then centrifuge these isolates at 40000 rpm for 10 min. After centrifugation pick supernatant for further use in PCR. Isolated DNA templates were stored at -20 °C.

Detection of carbapenem resistance genes by PCR:

Isolated DNA was amplified by using gene specific primer (Table1). A 25µL volume of reaction was prepared for PCR assay comprising 25mM MgCl₂ (2µl), buffer10X (2.5ml), Taq polymerase 1 unit, primers (forward and reverse) 10 pmole, template 1µl and distilled H₂O to make up the volume. The PCR conditions for amplification are given in (Table 2).

Primer name with target gene	Primer Sequence	Product size (bp)
16S rRNA	Forward, (5 GAAAGCCTGATCCAGCCATG-3) Reverse, (5-ACCACCCTCTACCGTACTCT-3)	293 bp
bla-IMP	Forward, (5 TGAGCAAGTTATCTGTATTC-3) Reverse, (5-TTAGTTGCTTGGTTTTGATG-3)	740 bp
bla-VIM	Forward, (5 -AGTGGTGAGTATCCGACAG-3) Reverse, (5-ATGAAAGTGCGTGGAGAC-3)	261 bp

Table1: Nucleotide sequences of primer for PCR amplification of genes

Target gene	First denaturation	Extension	Cycle: 30 annealing	Denaturation	Final extension
16S rRNA	95.0°C 5:00 min	95.0°C 0:45 sec	58.0°C 0:30 sec	72.0°C 0:30 sec	72.0°C 10 min
bla-IMP	95.0°C 5:00 min	95.0°C 0:45 sec	55.0°C 0:30 sec	72.0°C 0:30 sec	72.0°C 10 min
bla-VIM	95.0°C 5:00 min	95.0°C 0:45 sec	59.0°C 0:30 sec	72.0°C 0:30 sec	72.0°C 10 min

Table2: PCR programs for amplification of target genes

Results:

During the six month research period, 360 samples from different clinical labs were collected. Out of which 123 isolates of *Pseudomonas aeruginosa* were identified by biochemical tests, percentage of isolated *Pseudomonas aeruginosa* was as follows: urine 32 (35.5%), Pus 42 (46.6%), Sputum 30 (33.3%) and blood 19 (21.1%). following results of *Pseudomonas aeruginosa* antibiotic sensitivity were obtained, (Fig. 1) Gentamycin (59.00%), ciprofloxacin (55.00%) ceftriaxone (58.00%) ceftazidime (58.7%) cefotazime (57.0%) resistance rates to carbapenem group of antibiotics is doripenem (30.5%) meropenem (31.0%) and imipenem (28.0%). Out of 123 samples of *Pseudomonas aeruginosa* 28 isolates were found resistant to carbapenem group of antibiotic. Total 28 carbapenem resistant samples were processed for resistant gene (*bla-VIM* and *bla-IMP*) identification. All 28 samples were confirmed through internal control (16S ribosomal RNA) gene amplification (Fig. 2). Out of 28 positive control samples *bla-VIM* gene was detected in 04 samples and *bla-IMP* gene was identified in 09 samples (Table 3).

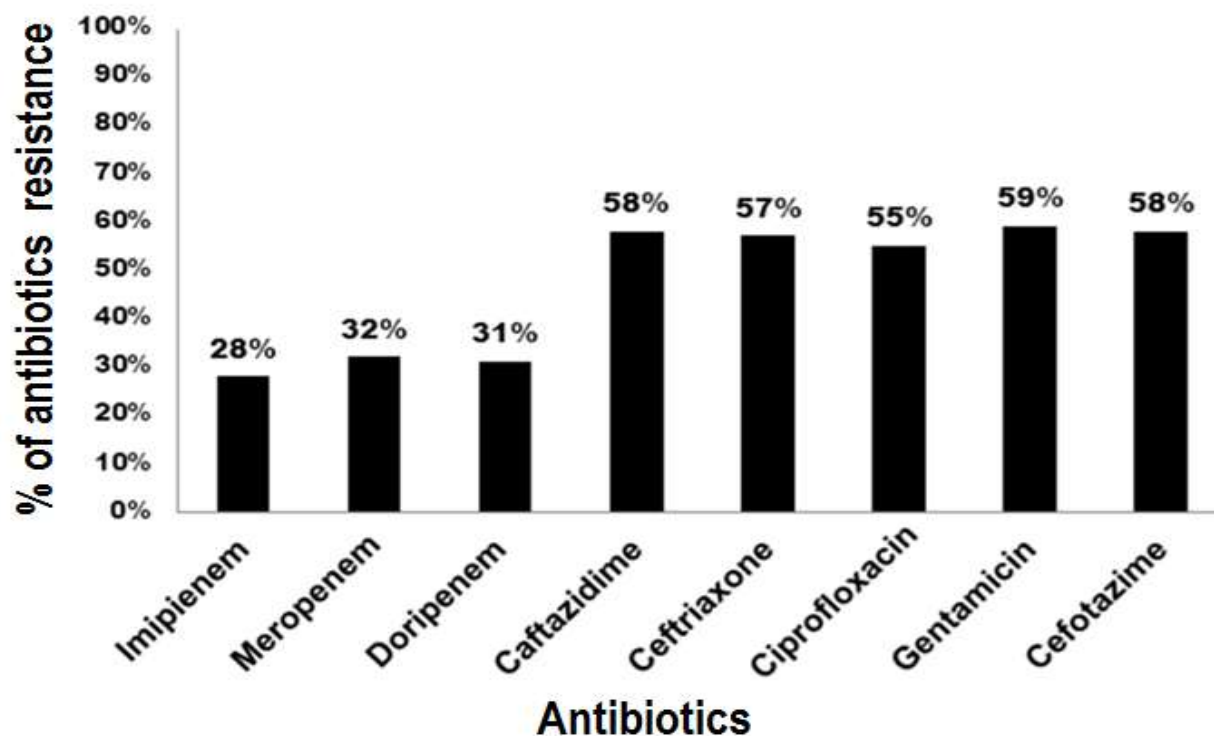


Figure 1: Graph displaying the percent antimicrobial resistance of *Pseudomonas aeruginosa* isolates.

Sr.#	Types of samples	Total No. of Samples	Total number of <i>Pseudomonas aeruginosa</i> isolates	Carbapenem resistance <i>P. aeruginosa</i>	Internal control positive samples	Bla-IMP positive samples	Bla-VIM positive samples	Both Bla-IMP and Bla-VIM
1	Urine	90	32	07	07	01	00	00
2	Pus	90	42	10	10	02	03	01
3	Sputum	90	30	07	07	01	05	01
4	Blood	90	19	03	03	00	01	00
Total samples		360	123	28	28	04	09	02

Table 3: Brief summary of results

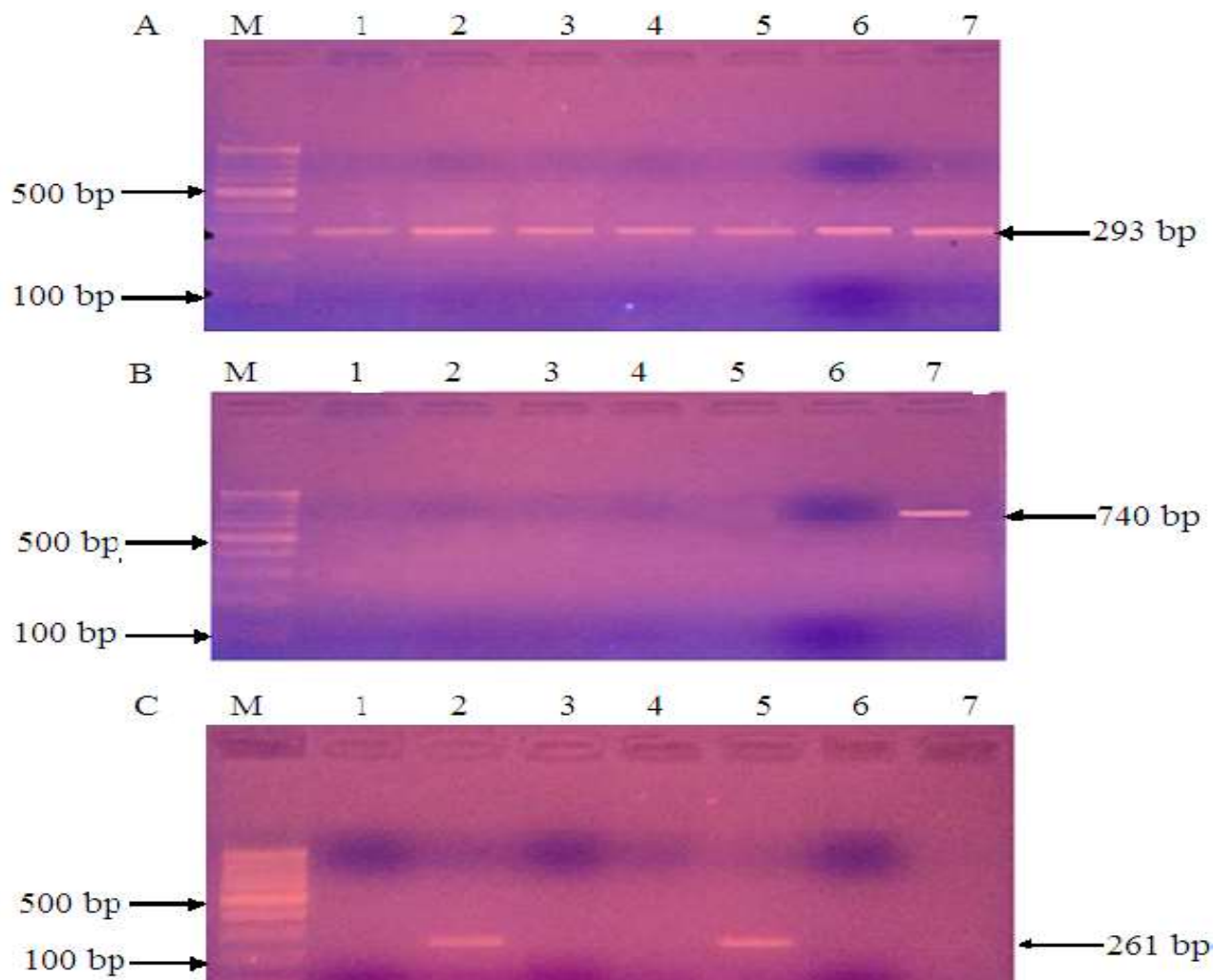


Figure 2: Gel electrophoresis showing amplified PCR product. **(A)** Represents PCR product of 16S ribosomal RNA gene of *Pseudomonas aeruginosa* as an internal control. **(B)** Represents the PCR products of IMP resistant gene. **(C)** Represents the PCR product of VIM resistant gene. Lane 'M' showing the 100 bp DNA ladder and lanes 1-7 are samples of *P. aeruginosa*

Discussion:

Pseudomonas aeruginosa is a major cause of morbidity and death throughout the world [11]. This organism is also creating health problems in Pakistan, in present study Urine, pus, sputum and blood samples were processed for the isolation of *P. aeruginosa*. Out of 360 samples, *Pseudomonas aeruginosa* was isolated from 35.5 % (32) urine samples, in Pus 46.6% (42) in sputum 33.3% (30) of and 21.1% (19) of the blood samples. In Karachi, Pakistan a study was conducted to isolate *Pseudomonas aeruginosa*. 5.4% isolates were found for *Pseudomonas aeruginosa* [12]. Same study was done in Jamnagar (India) to find *Pseudomonas aeruginosa* infection in hospitalized patient. 10.7 % samples were positive for *Pseudomonas aeruginosa* in urine samples [13]. In another study in Srikakulam (India) 9.28% pus samples were positive for *Pseudomonas aeruginosa* [14]. In Karachi another study was conducted *Pseudomonas aeruginosa* was isolated in 25% of sputum samples and 20% in blood samples [15]. Same study was done in Gujarat (India) *Pseudomonas aeruginosa* was found in 20% sputum samples and 13% in blood samples [16]. In our study a relatively high percentage of *Pseudomonas aeruginosa* was found in clinical specimen as compare to the studies mentioned above. Although, no clinical history of the specimens was collected but mostly in diagnostic laboratories these specimens were submitted with a complaint of infection and high ratio of pathogens from such samples is being anticipated.

In present study antibiotic susceptibility pattern of isolated *Pseudomonas aeruginosa* were tested against eight commercially prepared antibiotic. Doripenem, 31 %, imipenem 30%, meropenem 28%, cefotazime 58%, ceftazidime 48%, ceftriaxone 51%, ciprofloxacin 53% and gentamycin 61%. The highest resistant was found against gentamicin followed by meropenem. Same study was done in Rawalpindi (Pakistan), doripenem, 12 %, imipenem 09%, meropenem 14.6%, cefotazime 58%, ceftazidime 48%,

ceftriaxone 32%, ciprofloxacin 12% and gentamycin was found 80% resistant [17].

Multidrug resistant *Pseudomonas aeruginosa* is very difficult to treat but carbapenem are the most susceptible drugs for MDR treatment, now in recent years the increasing frequency of carbapenem-resistant *Pseudomonas aeruginosa* among the major problem. In our research carbapenem resistant *Pseudomonas aeruginosa* isolated from urine, pus, sputum and blood were 28.1 (9/32), 38.0% (16/42), 43.0% (13/30), and 26.3% (5/19) respectively. Same study was conduct in Karachi (Pakistan) carbapenem resistant *Pseudomonas aeruginosa* isolated from urine, pus, sputum and blood were 37.0% (12/32), 38.0% (16/42), 43.0% (13/30), and 26.3% (5/19) respectively [18]. An overall high percentage 22.7 % (28/123) of carbapenem resistant *Pseudomonas aeruginosa* were isolated in present study. Similar finding were recorded in Karachi 40% carbapenem resistant *Pseudomonas aeruginosa* were observed [19].

Over the last 20 years, many new β -lactam antibiotics have been developed that were significantly designed to be resistant against β -lactamases hydrolytic action. On the other hand, in the treatment of patients with each new class novel β -lactamases emerged that proved to be resistant against that class of drugs [20]. Among various mechanisms of resistance in *P. aeruginosa*, metallo-beta-lactamases is responsible for causing carbapenem resistant in *P. aeruginosa*.

16S rRNA primer was used to identify the pathogenic *Pseudomonas aeruginosa* all (28 samples) carbapenem resistant samples were positive for 16S rRNA. Same procedure was adopt in India to identify the *Pseudomonas aeruginosa* all isolates are positive for pathogenic *Pseudomonas aeruginosa* [21]. The IMP and VIM gene are responsible for producing metallo-beta-lactamases enzymes and producing resistant against most effective carbapenem drug. In our present study IMP and VIM was detected. Out of 28 positive carbapenem resistant samples IMP gene was detected in 09 (32.1%) samples and VIM

gene was detected in 04 (17.8%) samples. 02 samples shows both gene VIM and IMP. In Tehran (Iran) same study was conducted to evaluate the MBLs producing gene. Out of 32 samples IMP was detected in 18 (56.2%) samples and VIM was detected in 15 (46.2%) samples [22] The present study shows that the percentage of IMP and VIM gene is less in Pakistan as compare to Tehran (Iran).

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