Journal of Microbiology Research 2015, 5(6): 175-180
DOI: 10.5923/j.microbiology.20150506.02

Detection of *bla*$_{IMP}$ Gene in Metallo-β-Lactamase Producing Isolates of Imipenem Resistant *Pseudomonas aeruginosa*; an Alarming Threat

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Abstract *Pseudomonas aeruginosa* is one of the major cause of infections. There are two mechanisms by which *P. aeruginosa* has developed resistance against imipenem: by decreased drug permeability through the outer membrane proteins known as OprD and metallo-β-lactamase (MBL) production. Approximately 9 different types of MBL genes have been identified. Among them, *bla*$_{IMP}$ is the most prevalent one. Collected 200 laboratory isolates subjected for antimicrobial testing. Among them 28% (n =56) *P. aeruginosa* isolates were resistant to imipenem known as Imipenem Resistant *P. aeruginosa* (IRPA). Among all IRPA isolates; 87.5% (n=49) were found to be MBL producers. The detection of *bla*$_{IMP}$ gene was done after by ribotyping of IRPA strains. No *bla*$_{IMP}$ gene detection was present in all these MBL positive *P. aeruginosa*.

Keywords *P. aeruginosa*, IRPA, MBL, *bla*$_{IMP}$ gene

1. Introduction

Infectious diseases have affected the health of humans in many aspects and always remained one of the major causes of mortality and morbidity [1]. Amongst different microbial pathogens, *Pseudomonas aeruginosa* has been declared as one of the major cause of infectious agents belonging to the group of gram negative bacteria. It's an alarming threat to the hospitalized and immunosuppressed patients and has the ability to persist in different environmental conditions [2]. According to Center of Disease Control and Prevention (CDC), *P. aeruginosa* has been ranked as the fourth most commonly isolated nosocomial pathogen and its multidrug resistance poses a challenge for its treatment [3]. The emergence of imipenem resistance in *P. aeruginosa* has become a severe threat, especially in poor and developing countries due to the limitation of sources including Pakistan. The epidemiological data available in Pakistan indicates that the *P. aeruginosa* is one of the frequent pathogen cause infections in hospitalized patients. Other reports in Pakistan reported that in clinical isolates of *P. aeruginosa* an increase in percent resistance against imipenem was observed [4, 5].

Carbapenems and fluoroquinolones are potent and first line of therapy against *P. aeruginosa*. But now in some reported cases, *P. aeruginosa* has developed resistance against fluoroquinolones [6]. Carbapenems are the second choice of treatment and have been developed from thienamycin, a naturally derived product of *Streptomyces cattleya*. They inhibit L-D-transpeptidases during the cell wall synthesis. Among carbapenems known; imipenem is more important and effective antibiotic. It is the first-line of therapy against pseudomonal infections. It can bind strongly with penicillin binding proteins (PBPs), especially with PBP-2, PBP-1a and PBP-1b [7].

Resistance against imipenem knows as Imipenem Resistant *P. aeruginosa* (IRPA). There are two mechanisms by which *P. aeruginosa* has developed resistance against imipenem: by decreased drug permeability through the outer membrane proteins known as OprD and carbapenem hydrolyzing β-lactamase (metallo-β-lactamase). The resistance may be either by one or both of the two mechanisms [8]. The second major cause other is the metallo-β-lactamase (MBL) production. Metallo-β-Lactamase (MBL), according to the Ambler's classification is class B. It's a Zn^{2+} dependent carbapenem, active site of it have zinc ions for nucleophilic attack by zinc-bound water hydroxide. It is resistant to clavulanic acid but can be inhibited by EDTA (Ethylene-Diamine-Tetra-Acetic Acid), Tetramethylethylenediamine and other chelating agents [9].

MBL was first detected in *Bacillus cereus,* in 1960s and they have intrinsic chromosome resistance against β-lactam antibiotics. Approximately 9 different types of MBL have been identified in gram negatives but most common and

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Published online at http://journal.sapub.org/microbiology

important types of acquired MBLs include IMP (Imipenem Carbapenemase) (Kyorin Health *S*cience MBL 1) is rare [10, 11]. In 1991, first plasmid-mediated MBL gene, IMP-1 was identified in *P. aeruginosa* from Japan [4], then subsequently other IMP-type of MBLs were detected in other Eastern countries, including Europe, Canada and Brazil. Analysis of gene bla_{IMP} showed the features of different class integrons. Integrons are DNA-based structures that carry the genes of antibiotic resistance, known as gene cassettes and most transferable MBLs are encoded by type 1 and 3 integrons [12, 13].

Objectives of Study: Main objective of this research is to investigate the prevalence of Imipenem Resistant *P. aeruginosa* (IRPA) and detection of bla_{IMP} gene in the confirmed cases of MBL strains.

2. Material and Methods

2.1. Study and Location

Our study was carried out in the Government College University, Lahore and King Edward Medical College Lahore.

2.2. Bacterial Isolation and Identification

Two Hundred laboratory samples were collected and inoculated on MacConkey and Blood agar for the isolation of *P. aeruginosa*. Initial identification was done phenotypicaly including API 20NE galleries (BioMerieux, France).

2.2.1. Antibiotic Sensitivity Test and Detection of Imipenem Resistance

Antimicrobial sensitivity testing was performed by according to CLSI recommendation 2012, in order to investigate the presence of imipenem resistance in *P. aeruginosa*.

2.2.2. Detection of MBL

IRPA are MBL producers. Confirmation of metallo-β-lactamase production was done by imipenem-EDTA combined disk diffusion method. MBL need Zn^{2+} at its active site for activation. Whereas, EDTA as a chelating agent to inactivate the MBL by capturing the zinc ions. EDTA solution of 0.5 M (750 µg EDTA) concentration was prepared in this method by adding 186.1 g of di-sodium EDTA. $2H_20$ (Sigma) in 1 liter distilled water. NaOH was added to adjust the pH of solution 8.0. This solution was sterilized by autoclaving. On the dried plate of Mueller-Hinton agar two imipenem (10 µg) disks (Oxoid, UK) were placed. Out of two imipenem disks, on one disk 4 µl volume of 0.5 M EDTA was added. Plates were incubated for 18-20 hours at 35°C. EDTA causes an increase in the zone of inhibition from 8-15 mm (imipenem plus EDTA) were regarded as MBL producers, whereas, an increase in the zone of inhibition from 0-5 mm was regarded as MBL non-producers [14] (as shown in the Fig. 1).Positive and negative controls as *P. aeruginosa* (MBL positive) and *P. aeruginosa* (MBL negative) respectively were also included.

2.2.3. Ribotyping

All the imipenem resistant MBL positive *P. aeruginosa* isolates were re-confirmed by ribotyping of 16S rRNA by polymerase chain reaction (PCR) and amplified product was isolated on 1% agarose gel electrophoresis. The forward and reverse primers are utilized for PCR reaction was as follows [15]:

Forward Primer: 5' -AGA GTT TGA TCC TGG CTC AG -3'

Reverse Primer: 5' –TAC GGT TAC CTT GTT ACG ACT T- 3'

The correct size of end product and for the separation of mixed population of DNA was determined by running the product on 1% agarose gel.

2.2.4. Phylogenetic Analysis

Phylogenetic trees, based on homology search results through BLAST [16] were constructed taking the 16S rRNA sequences of each of IRPA sequence as query. As the 16S RNA is very much conserved in all of the bacteria, therefore, during BLAST search the option of 'similar sequences' was chosen instead that of 'dissimilar sequences' so as search may include more diverse sequences to find the Phylogenetic relationship of 16S RNA sequence of IRPA isolates of this study. Moreover, 16S ribosomal RNA sequences database was selected for the homology search through BLAST at NCBI. The Phylogenetic trees of relationship were through neighbor joining method using percent identity through Jalview [17].

2.2.5. Detection of bla_{IMP} Gene

For the detection of bla_{IMP} gene, PCR and 1% agarose gel electrophoresis was performed on MBL producing isolates of P. *aeruginosa.* The primers utilized for PCR reaction were as follows [18].

***bla*$_{IMP}$** Forward Primer: 5' - GAA GGY GTT TAT GTT CAT AC-3'

***bla*$_{IMP}$** Reverse Primer: 5' - GTA MGT TTC AAG AGT GAT GC - 3'

The correct size of end product and for the separation of mixed population of DNA was determined by running the product on 1% agarose gel.

2.2.6. ATCC Strain

ATCC 27853 *Pseudomonas aeruginosa* and were included to monitor quality control.

2.2.7. Statistical Analysis

Data was analyzed by using computer software program SPSS 19.0. The values are expressed in percentages. Chi square test was used for the determination of the statistical significance (p-value).

3. Results and Discussion

In the present study, we have collected 17 different categories of clinical samples (Table 1). The percentage of the indoor patients 70.5% (n=141), having *P. aeruginosa*, were observed to be much higher compared to the outdoor patients 29.5% (n=59). These findings are similar to the study of Prashant *et al*., in 2011 [19]. According to them, indoor patients, with *P. aeruginosa*, were 84.92% compared to the outdoor patients (15.07%). These results correspond to the fact that duration of hospital stay has been related with incidence and prevalence of *P. aeruginosa* infections.

Table 1. List of Collected Clinical Samples having *P. aeruginosa* infection

Sr. No	Sample	Number	Percent
1	Pus	69	34.5
2	Wound Swab	52	26
3	Sputum	14	7
4	Pleural Fluid	13	6.5
5	Ear Swab	12	6
6	Tips	10	5
7	Urine	7	3.5
8	BLOOD	4	2
9	TISSUE	4	2
10	Ascitic Fluid	3	1.5
11	Bronchial Washing	3	1.5
12	HVS	3	1.5
13	Semen	2	1
14	CSF	1	0.5
15	Endometrial Fluid	1	0.5
16	Pelvic Drain	1	0.5
17	Peritoneal Fluid	1	0.5
	TOTAL	200	100

Some *P. aeruginosa* isolates were found as either a single pathogen 40% (n=80) or in the combination with other organisms (polymicrobial) 60% (n=120). Some isolates have produced a blue-green pigment (pyocyanin) 72% (n=144). Finlayson in 2011 concluded that production of pigment significantly associated with the multidrug resistance and also the virulence in *P. aeruginosa* [20]. MBL production also affected by pigment production; according the statistical analysis; p-value (probability significance) was found less than the targeted value ($p<0.05$) as $p=0.021$ (Table 2).

Table 2. Association of Pigment Production with MBL Production

Pigment Production	MBL		Total	
	Positive	Negative		
Pigmented	29	115	144	p=0.021
Non-Pigmented	20	36	56	
Total	49	151	200	

Resistance against imipenem was found in 28% (n=56) IRPA isolates whereas 72% (n=144) were sensitive (Fig. 1). Different percentages of imipenem resistance were found in different regions of world. In 2010, Akhtar *et al.* observed 26.1% IRPA in Pakistan [21]. Prashant *et al.* in 2011 had found 12.69% IRPA in India [19]. In 2012, Fatimah *et al.*, have found imipenem resistance in 24% isolates of *P. aeruginosa* in Karachi, Pakistan [22], Zoghlami *et al.* observed the imipenem resistance in 37.1% isolates of *P. aeruginosa* in Tunisia [2] and Dong *et al.,* observed 15% IRPA in Taiwan [23]. In 2013, Morrow *et al*. observed 78.1% IRPA in USA [24]. The data shows an increase in percentage of resistance in *P. aeruginosa* which is an alarming threat.

Main culprit for the imipenem resistance is the production of Metallo-β-lactamase (MBL). MBL was detected in 87.5% (n=49) of IRPA isolates (n=56). Prashant *e al.,* 2011 in India, determined the MBL in 62.5% isolates of imipenem resistant *P. aeruginosa* [19]. Vahdani *et al.* in 2012 have observed 38% MBL positivity in burns, Iran [25].

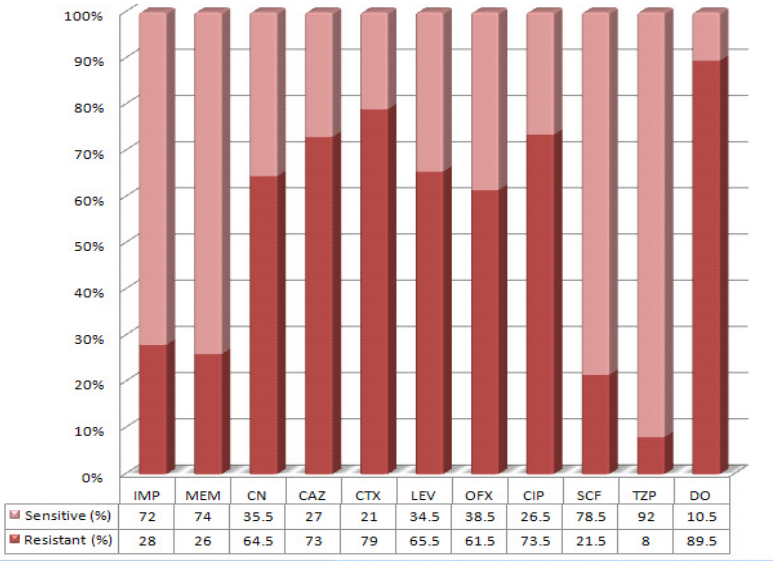

Figure 1. Resistance Pattern of 200 isolates of *P. aeruginosa* against various antimicrobials (%age)

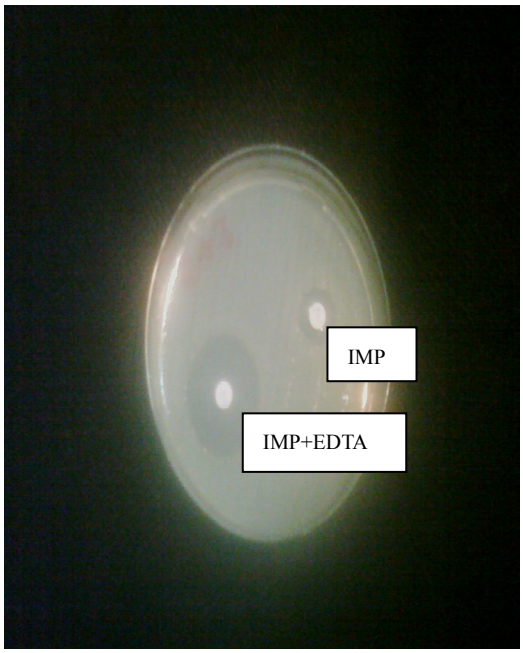

Figure 2. MBL Detection by Imipenem-EDTA Disc diffusion Method

Re-confirmation of all the MBL positive *P. aeruginosa* isolates was done by ribotyping for further bla_{IMP} gene detection. After ribotyping of 16S rRNA, a product gene around 500bp was produced and all were found to be *P. aeruginosa* confirmed by gene sequencing of the end product. Sequencing of 16S rRNA of all IRPA isolates resulted in three different lengths of sequences. Of the total 49 IRPA isolates 23 (47%) specimen were with a gene length of 1461 bp whereas, 9 were with a sequence length of 1456 bp and remaining 7 isolates resulted in a length of 1326 bp. Homology search results and phylogenetic analysis revealed that all IRPA isolates of the present study were very much related to *P. aeruginosa* PAO1 strain (Figure 3).

The confirmed isolates of MBL positive *P. aeruginosa* were evaluated by PCR to detect bla_{IMP} gene, end product of 230 bp was produced (Figure 4) which was compared with 1Kb DNA Ladder. This gene product was much smaller than our desired product of 587 bp of bla_{IMP} gene. It confirmed that bla_{IMP} gene was not found in any of the isolate resistant to imipenem.

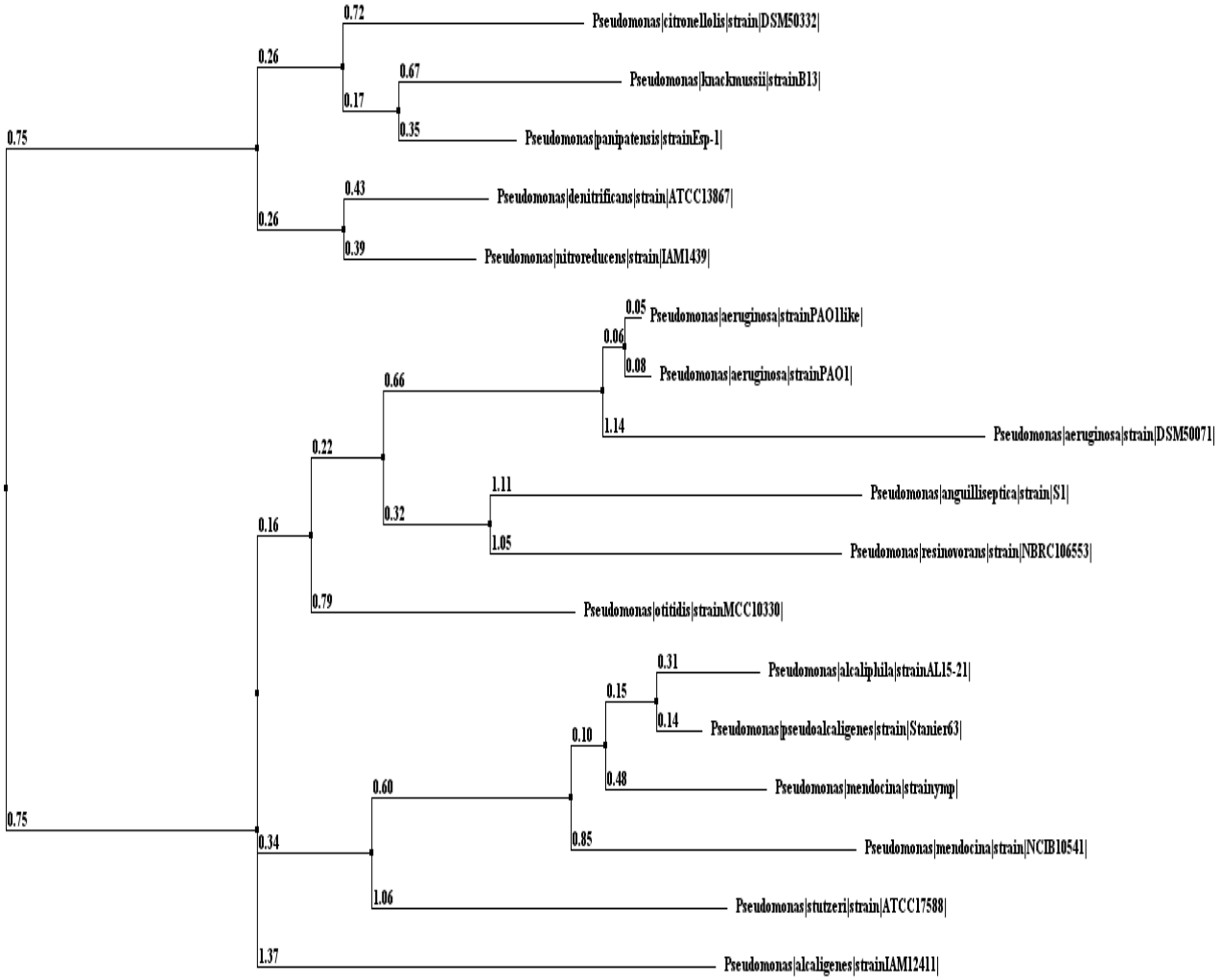

Figure 3. Phylogenetic Tree

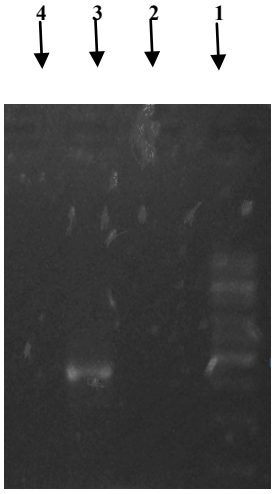

Figure 4. 1Kb DNA Ladder, 2-4: Test isolates for bla_{IMP} detection

Imipenem resistance either by decreased drug permeability (OprD) or MBL production both can be present simultaneously or individually [8]. Amongst 9 different categories of *bla* genes bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{NDM}, and bla_{KHM-1} were found common and important ones [10]. bla_{IMP} was not detected in the present study; the reasons behind it may be the decreased drug permeability or deficiency of OprD or any type of MBL gene production. Data regarding investigation on MBL gene detection in Pakistani is limited and only one study reported was on bla_{IMP-13} and OprD in *P. aeruginosa*, in Karachi [26].

The current study is in line with Mohammad *et al*. in 2008 in Iran; they have detected 23 imipenem resistant isolates of *P. aeruginosa* in Iranian burn center of level I, but did not found the bla_{IMP} gene in their hospital settings [15]. Khosravi *et al*. in 2011 (Malaysia) have collected 90 IRPA isolates, 32 were MBL positive whereas bla_{IMP} was found in 14, bla_{VIM} in 18, bla_{IMP-7} in 12, bla_{IMP-4} in 2, bla_{VIM-2} in 17 and bla_{VIM-11} in 1 isolates of MBL producers in IRPA isolates [27] . In 2011, Mereuta *et al.* observed bla_{VIM} in MBL isolates of IRPA but absence of bla_{IMP} in Lasi [28]. Jacome et al. (2012) in Brazil, did not found the blaIMP or blaVIM genes but blaSMP-1 was detected from 44.8% of MBL producers [29]. In the end, blaIMP gene has not been found in our isolated strains of MBL producing P. aeruginosa. Reason behind it might be the production other *bla* genes or OprD deficiency.

4. Conclusions

Overall, 28% IRPA has been observed in this study which is an important issue regarding the treatment of infection. Drugs including Tazocin and Sulzone have been found to be the most effective antimicrobials against IRPA. However no bla_{IMP} gene has been found in our hospital settings but still scope of detection for the presence of MBL gene in Pakistani IRPA isolates is much high.

ACKNOWLEDGEMENTS

We are grateful to kind Edward Medical College, Mue Hospital Lahore for providing us the clinical materials for analysis and with special thanks to the University of Punjab Lahore, for the use of their research facilities and to all the participants.

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