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Research Article

Molecular Analysis of Metallo Beta Lactamase in Multi Drug Resistant *Pseudomonas Aeruginosa* among the Clinical Isolates

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Keywords *Pseudomonas Aeruginosa*; Metalloid β-lactamase; Genes; BlaVIM-2; PCR

Abstract

Background: One of the major clinical problems regarding Pseudomonas aeruginosa is attributed to the production of Metallo-Betalactamase (MBL) enzymes. This group of enzymes is members of beta-lactamases which constitute Ambler class B that hydrolyze-carbapenems. This study was carried out to find out the predominant resistance mechanisms among MDRPA and the prevalence of corresponding resistance genes.

Materials and methods: MDRPA isolates collected from various clinical samples for a period of one year from March 2015 to February 2016 were included to detect the predominant mechanism of resistance using phenotypic and molecular methods. Molecular characterization of all these isolates was done by Polymerase Chain Reaction (PCR) for the presence of blaVIM2, blaIMP-1, blaOXA-23, and blaNDM-1 genes with specific primers.

Results: Among 120 MDRPA isolates 70 (58.33%) were MBL producers. Molecular characterization studied by PCR showed 15 (12.5%) of vim2 gene and only 2 (1.66%) of IMP 1 gene. None of the 120 MDRPA has produced OXA 23 and NDM gene in our study.

Conclusion: The prevalence of MBLs has been increasing worldwide, particularly among P. aeruginosa, leading to severe limitations in the therapeutic options for the management. Thus, proper resistance screening measures and appropriate antibiotic policy can be strictly adopted by all the healthcare facility providers to overcome these superbugs.

Introduction

Pseudomonas aeruginosa is one of the most important pathogens causing nosocomial infections, it is naturally resistant to many antimicrobial agents. It has a distinctive capacity to become resistant to many available antimicrobial agents via multiple mechanisms [1]. In the past decade, acquired multidrug resistance, relating to selective antibiotic pressure, has emerged in several countries; and in some cases, infections caused by multidrug resistant *P. aeruginosa* have been untreatable [2]. Carbapenems, including meropenem and imipenem, are the most effective antibiotic against this organism isolated from patients. However, resistance to carbapenems has emerged by different mechanisms such as impermeability to drug due to loss of Oprd porin, the up-regulation of an active efflux pump system present in the cytoplasmic membrane of these organisms or production of Metallo-Beta-Lactamases (MBLs) that hydrolyze all carbapenems [3,4]. As carbapenems are the potent antimicrobial weapon against Multi Drug Resistant *P. Aeruginosa* (MDRPA), this bacterium has developed resistance even against this group of drugs by producing MBLs (carbapenemase) [5]. Imipenem and meropenem among carbapenems have gained increased therapeutic access in many medical centers against MDRPA. However, as this pathogen has gained already resistance even to these available drugs, identification of nosocomial strains capable of producing MBL has aroused more interest and importance in the recent years [6].

Carbapenemases are Class B MBLs; IMP, VIM or Class D oxacillinases (*OXA* 23-*OXA* 27) (carbapenem-hydrolyzing Class D β-lactamases or Class A clavulanic acid inhibitory enzymes (SME, NMC, IMI, KPC). Class A β-lactamases with activity again carbapenems, are uncommon and divided into five groups (GES, IMI, KPC, NMC-A and SME). ESBLs and Carbapenemases are typically encoded by plasmid or transposon-borne genes, often on integron, which are genetic elements capable of capturing and subsequently mobilizing resistance genes, although some β-lactamase genes are associated with novel mobile insertion sequences termed insertion sequence common region elements [6]. Acquired MBLs includes the VIM and IMP enzymes, of which there are numerous variants of the original VIM-1 and IMP-1 MBLs as well as the SPM-1, GIM-1, NDM-1, AIM-1 and SIM-1 enzymes [7,8]. The VIM and IMP enzymes are by far the most common MBLs found in carbapenem-resistant bacteria, including carbapenem-resistant *P. aeruginosa* [9]. Thus,

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Table 1: List of primers used.

Primer name	Sequence (5' to 3')	Amplicon size (bp)
bla VIM-2	Forward- ATGTTCAAACTTTTTGAGTAGTAAG Reverse - CTACTAACGACTGAGCG	**801**
bla IMP	Forward - CTACCGCAGCAGAGTCTTTGC Reverse - GAACAACCAGTTTTGCCTTACC	**640**
blaOXA-23	Forward - GATGTGTCATAGTATTCGTCGT Reverse - TCACAACAACTAAAAGCACTGT	**1058**
blaNDM-1	Forward - GGTTTGGCGATCTGGTTTTC Reverse - CGGAATGGCTCATCACGATC	**621**

this study was conducted to know the prevalence of MBL producing multidrug resistant *P. aeruginosa* and the molecular characterization of prevalent genes present in them in order to improve the therapeutic options and to decrease the morbidity and mortality.

Materials and Methods

The present study was carried out on *P. aeruginosa* obtained from various clinical samples from clinical laboratories during the period of March 2015 to February 2016. One hundred and twenty MDR *P. aeruginosa* isolates were obtained from 400 clinical samples The samples from which the strains were isolated include blood, Pus, Urine, Broncho Alveolar Lavage (BAL) and Endotracheal (ET) aspirates and tissues. All the samples were processed for isolation and antibiotic sensitivityby Kirby Bauer method. All clinical isolates of *P. aeruginosa* which were found to be multidrug resistant (total of 120 MDRPA) were included in this study for further characterization. Repeated isolates from the same patients were excluded.

Detection of MBL producing *P. aeruginosa* was performed by the **Imipenem- EDTA Combined Disc Test (CDT)**: The CDT was performed as described by Yong et al. [10] and Molecular characterization for these isolates were done by Polymerase Chain Reaction (PCR) for the presence of *VIM*2, *IMP* 1, *OXA* 23 and *NDM* 1 genes. ATCC *P. aeruginosa* 27853 was used as a control strain for all the procedures.

Polymerase chain reaction amplification of resistance genes

The genomic DNA was extracted from all the 120 MDRPA isolates followed by agarose gel electrophoresis. The isolated DNA was used as a template for amplification of specific genes described below. PCR amplification was done with specific gene primers and checked for the presence of the corresponding gene responsible for MBL production such as bla_{VIM-2}, bla_{IMP-1}, bla_{OXA-23}, and bla_{NDM-1} genes. The PCR amplification was performed with Eppendorf Master Cycler.

Amplification of the resistant genes were carried out with the following reaction mixture composition (10µl): DNA template (50 ng), 1µl each of deoxynucleotide triphosphates (2.5µM), Taq buffer (10µl), forward and reverse primers (2.5µM) and 1 U of Taq DNA polymerase (Merck Biosciences, Darmstadt, Germany) (Table 1). All PCR amplifications were performed using thermal cycler (Veriti Thermal cycler, Applied Biosystems, USA.) using the following conditions for 30 cycles: 94°C for 5 min, 94°C for 45 s, annealing at 54°C for 30 s and extension at 72°C for 45 s. The PCR products were analyzed on 1.5% agarose gel, stained with ethidium bromide, and the amplicons were purified using HiPura PCR product purification kit (Himedia, Mumbai, India).

Table 2: Antibiotic resistance among isolates of MDR *P. aeruginosa.*

ANTIBIOTICS	RESISTANT	SENSITIVE	INTERMEDIATE
piperacillin (75µg)	74 (61.66%)	46 (38.33%)	0 (0%)
piperacillin-tazobactam (100/10µg)	59 (49.1%)	54 (45%)	7 (5.83%)
ceftazidime(30µg)	96 (80%)	15 (12.5%)	9 (7.5%)
cefoperazone(75µg)	92 (76.66%)	9 (7.5%)	19 (15.83%)
ceftriaxone(30µg)	98 (81.66%)	9 (7.5%)	13 (10.83%)
imipenem(10µg)	55 (45.83%)	65 (54.16%)	0 (0%)
meropenem(10µg)	65 (54.16%)	54 (45%)	1 (0.8%)
gentamicin(10µg)	85 (70.83%)	35 (29.16%)	0 (0%)
amikacin(30µg)	63 (52.5%)	44 (36.66%)	13 (10.83%)
ciprofloxacin(5µg)	112 (93.33%)	4 (3.3%)	4 (3.3%)
cefoperazone-sulbactam(75/25µg)	87 (72.5%)	11 (9.16%)	22 (18.33%)
tobramycin(10µg)	101 (84.16%)	19 (15.83%)	0 (0%)
netilmicin(30µg)	66 (55%)	54 (45%)	0 (0%)
gatifloxacin(5µg)	95 (79.16%)	18 (15%)	7 (5.83%)
PolymyxinB(300U)	00 (0%)	120 (100%)	0 (0%)

Statisticalanalysis

All the data were entered in Microsoft Excel sheet and the results were analyzed by SPSS software. (IBM, USA)

Results

A total of 120 MDRPA, carbapenemase resistance was documented among 54.16% (65) towards meropenem and 45.83% (55) towards imipenem, respectively. All these 120 isolates showed 100% susceptibility towards polymxin B by Kirby Bauer disc diffusion method. Antibiotic resistance among isolates of MDR *P. aeruginosa* reflected in Table (2), Figure (1). The Minimum Inhibitory Concentration (MIC) of meropenem for resistant strains ranged from 8 µg/ml to >64 µg/ml. (Break point MIC-≤4 µg/ml to ≤16 µg/ml.

Amongst these 120 MDR *P. aeruginosa*, seventy isolates (58.33%) were found to be MBL producers. Fifteen (12.5%) of MDRPA showed presence of *Vim*2 gene and only 2 (1.66%) was positive for *IMP* 1

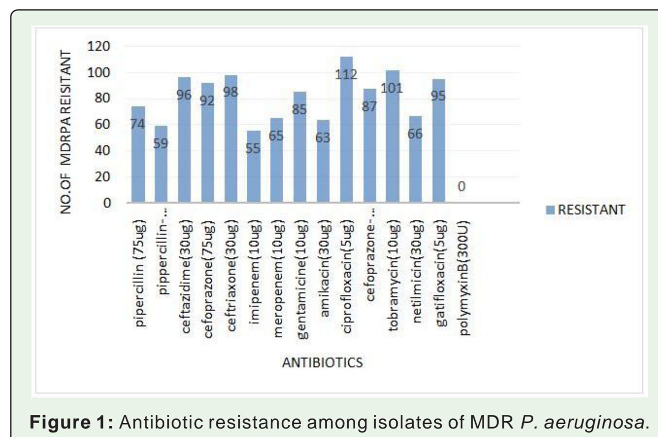

Figure 1: Antibiotic resistance among isolates of MDR *P. aeruginosa.*

gene. None of the 120 MDRPA have produced *OXA* 23 and *NDM* gene in our study.

Discussion

The rapid spread of MBLs among major gram-negative pathogens, particularly *P. aeruginosa*, is an emerging threat and a matter of concern worldwide [11,12]. These organisms are resistant to almost all commonly available antibiotics with limited treatment options. Thus, this study was conducted to know the prevalence of MBL producing multidrug resistant *P. aeruginosa* and the molecular characterization of prevalent genes present in them in order to improve the therapeutic options and to decrease the morbidity and mortality.

In the present study, MDRPA isolates showed the resistance to carbapenems like meropenem (54.16%) and imipenem (45.83%), which were found to be the precious weapon against MDRPA infections and this is an alarming sign. All the isolates showed 100% sensitive to polymyxin B. In 2014, a study by Samira *et al* 2014 reported, imipenem and meropenem resistance was observed to be fifty five isolates (45.83%) and sixty five isolates (54.16%) respectively which is more likely case in our study [13]. In 2008, a study by Alis, Kan et al. with 1071 MDRPA, reported resistance to imipenem (22.5%) and meropenem (31%) [14]. Deepak *et al.* during 2009 to 2010 with 193 *P. aeruginosa* reported resistance to imipenem (3.7%), which is less compared with the present study [15]. Minimum Inhibitory concentration of meropenem ranged from 8μg/ml to >64μg/ml, which is comparable to other studies. About 63.33% of these MDRPA isolates showed higher MIC to meropenem. In 2006, Shashikala et al. carbapenem resistant *P. aeruginosa* had reported MIC ranging from 8μg/ml to 64μg/ml [16]. A study by Fernandez *et al.* In 2010, Higher MIC of 128μg/L for meropenem got documented [17]. All these resistance ranging pattern is mostly directly dependent on various factors, which mainly includes the antibiotic policy in practice in the respective healthcare setups.

As MBLs production is the major mechanism of resistance among MDRPA, A study by Jayakumar *et al* in 2007. Reported 54.5% MBL producers [18]. Morten *et al* in 2001 have reported 47% of MBL producers in *P. aeruginosa* [19]. Upadhyay *et al* in 2010. Reported 46.6% of MBL production among MDRPA strains [20]. Poirel L *et al* in 2000 reported 36% of MBL producers among the MDRPA [21]. Navaneeth *et al* in 2002. Reported 12% MBL production in *P. aeruginosa* [22]. Another recent study by Varaiya *et al* in 2008. Showed 20.8% of MDRPA were found to be MBL producers [23]. In comparison with our present study, we reported a high prevalence of 58.33% of MBL producers. The Remaining MDRPA strains may be harbor in some other resistance mechanism like, ESBL production, Amp C production, bio film formation or through various virulence factors.

In *P. aeruginosa* number of different β-lactamases has been described including MBL, ESBL and *OXA* production. This present study investigated the predominant β-latamase coding genes such as, *VIM*-2, *IMP*-1, *OXA*-23 and *NDM*-1 through PCR. Among MBL producing isolates in our study, the presence of *VIM*-2 gene is predominant when compared with *IMP*-1 MBL gene. Surprisingly, none of our isolates were positive for *OXA*-23 and *NDM* genes. The presence of *VIM*-2 gene appears to be more prevalent in our study.

In 2007, the first case of MBL gene bla_{VIM-2} was reported in a strain isolated in India. This VIM2, which is present on integron had its ancestral Class I integron documented in United States and Russia. [24-26]. This Class I integron having 3' conserved sequence have arisen from an ancestral integron predating the formation of 3' conserved, which was found in United States and Russia. The present study documented occurrence of bla_{VIM-2} among its collection, wherein 15 isolates of its collection were positive for bla_{VIM-2}. Interestingly, the DNA sequence analysis of all our VIM-2 showed 100% identity with the sequence of global genotypes retrieved from the Gen Bank public database. This finding suggests successful global dissemination of VIM-2 resistant gene that is of great concern. *VIM*-2 gene was found to be more prevalent among MDRPA in our study as revealed by PCR method.

Fortunately in our study we encountered very less prevalence of resistance genes among our *P. aeruginosa* isolates when compared to rest of the world, wherein, high incidence of MBL have been reported. From the results obtained through our investigation, it can be concluded that VIM2 gene was the most frequently isolated β-lactamase gene among the *P. aeruginosa*. The sequencing results further confirmed, there is less variance among our β-lactamase genes when compared to global genotypes.

MDRPA infections are likely to affect critically ill patients who require prolonged hospitalization. Infections with MDRPA are also associated with adverse clinical outcome. Strict isolation of patients infected with MDRPA and judicial use of antibiotics should be emphasized in order to prevent the spread of MDRPA infections. Further, more clinical studies are needed to identify risk factors for MDRPA development and to determine the economic impact of these infections, as well as to determine the most efficacious antimicrobial regimens and duration of therapy to maximize the outcome of MDRPA infections.

Conclusion

The present study gives the alarming sign toward the high prevalence of carbapenem resistant nonfermenting pathogens. Thus, this calls for stringent preventive measures, which includes strict infection control practices and judicious use of antibiotics with implementation of antibiotic policy. These kinds of important measures might overcome the challenge of high mortality posed by MDRPA and other nonfermenting bacterial pathogens.

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