"Molecular Characterization of Blaimp-1 Gene and Identification of Metallo-Beta-Lactamase Producers among Carbapenem Resistant Pseudomonas Aeruginosai isolated from Hospitalized Patients in a Tertiary Care Hospital, Kanpur"

R. Sujatha[1], Nashra Afaq*[2], Deepak Sameer[3]

Abstract:

Introduction: Pseudomonas aeruginosa has the ability to persist in both community as well as the hospital settings. It is an important cause of multidrug-resistant nosocomial infections. Carbapenems are the drug of choice for the infections caused by Pseudomonas aeruginosa but worldwide emergence of carbapenem resistance Pseudomonas aeruginosa including resistance to beta-lactams, Aminoglycosides, and Fluoroquinolones represents an extraordinary threat to public health.

Aim and Objectives: To study the Molecular Characterization of blaIMP-1 gene and study of metallo-beta-lactamase producers among carbapenem resistant Pseudomonas aeruginosa isolated from Hospitalized Patients in a Tertiary care hospital, Kanpur.

Material and Methods: This was a cross sectional study conducted in the Department of Microbiology, in a Rama medical college hospital & Research centre Kanpur, over a period of one year from March 2021 to March 2022. Ethical clearance was duly obtained from the Institute Ethical Committee for conducting the study. A total of 200 clinical samples were included in our study. The direct microscopic examination and biochemicals test for identification of P. aeruginosa was done. The isolates were then subjected to antimicrobial susceptibility testing by KirbyBauer disk diffusion method according to CLSI guidelines. All positive isolates were further tested by Imipenem- Ethylene diamine tetraacetic acid combined disc test and Modified Hodge Test (MHT) for the MBL detection. Isolates tested positive in the phenotypic test are subjected to conventional PCR for detection of the gene coding for MBLs. The DNA Extraction of the test isolates was carried out followed by the PCR.

Results: Out of 200 clinical isolates, there was 86 (43%) isolated from urine, 37 (18.5%) from trachea, 32 (16%) from lesion, 27 (13.5%) from blood, 18 (9%) from pus. There was 130 isolates which was found resistant to imipenem. Of the 130 imipenem resistant isolates, 108(83%) was MBL producer as determined by CDDT. All MBL producing isolates was found resistant to the examined antibiotics. The results of amplified genes by PCR showed that 28 MBL-producing isolates contained blaIMP-1.

Conclusion: The screening for MBL production in microbiology laboratories is very important for the treatment of patients, specially hospitalized patients and also to prevent the possible spread of resistance to other Gram-negative organisms because of their broad-spectrum drug resistance which creates a therapeutic challenge to the clinicians.

Keywords: Carbapenems, MHT, MBL, broad-spectrum drug . DNA, PCR,

Introduction

Pseudomonas aeruginosa originally is an environmental bacterium that is considered as an opportunistic pathogen which infects hospitalized and immune-compromised patients of the many different types of Pseudomonas, the one that most often causes infections in humans is called Pseudomonas aeruginosa, which can cause infections in the blood, lungs (pneumonia), or other parts of the body after surgery.

Professor[1] & Head[*], Dept of Microbiology Rama Medical College Hospital and Research Centre, Mandhana Kanpur
Research Associate[2], Dept of Microbiology Rama Medical College Hospital and Research Centre, Mandhana Kanpur*
Tutor[3], Dept of Microbiology Rama Medical College Hospital and Research Centre, Mandhana Kanpur.

The carbapenem-resistant P. aeruginosa causes serious infections, such as nosocomial pneumonia which is increasing in the hospitalized patients [1]. Resistance to carbapenems is often associated with production of metallo-ß-lactamases [1, 2]. The most effective antibiotics that can be used against Pseudomonas aeruginosa are ß-lactam antibiotics in which impanel as a carbapenem is considered as the most appropriate antibiotic to be used against the mentioned organisms because of emergence of multidrug-resistant strains [2]. Carbapenem resistance occurs because of decrease in antibiotics absorption due to lack of an outer membrane porin, as oprD, exclusion from the cell by efflux pump [3].

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decrease in outer membrane permeability and production of MBL [2, 3]. Nowadays the emergence of antibiotic resistance strains is one of the challenges in treating patients, such as MBLs producing Pseudomonas aeruginosa [4, 5].

Carbapenemases have two main molecular families' serine carbapenemases and other metallo-carbapenemases, which are considered as subgroup of metallo-beta-lactamases (MBLs). The VIM, IMP and SPM types are the most clinically significant carbapenemases which encoded by blaIMP, blaVIM and bla SPM genes [6]. At least 14 different VIMs and 23 different IMP MBLs have been identified so far. MBLs also divided into several families as follows: IMP, VIM, SPM, GIM, SIM, DIM, AIM, KHM, NDM and KPC. Most of them, if not all, genes encoding IMP, VIM and SPM types as well as GIM are found as gene cassettes in class 1 integrons, although IMP MBL genes are also found on class 3 integrons [6,7].

There is a need for immediate detection of MBL-producing P. aeruginosa as to prevent the spread of the organism within and between hospitals, and to accurately treatment infections caused by this bacterium. Therefore, this study is undertaken for the Molecular Identification of metallo-beta-lactamase producers among carbapenem resistant Pseudomonas aeruginosa isolated from Hospitalized Patients in a Tertiary care hospital.

Material and Methods

This was a cross sectional study conducted in the Department of Microbiology, Rama medical college hospital & Research centre Kanpur, over a period of one year from March 2021 to March 2022. Ethical clearance was duly obtained from the Institute Ethical Committee for conducting the study. A total of 200 clinical samples were included in our study. Theses bacteria had been isolated from different clinical specimens such as urine, wound, blood, trachea and other clinical specimens. After transporting the samples to the microbiology laboratory in Medical School, the colonies were again inoculated into MacConkey agar medium and pure colonies were identified as P. aeruginosa based on Gram staining and biochemical tests such as oxidase, catalase, Oxidative-fermentative test, growth on media such as TSI, SIM, cetrimide agar and growth at 42°C [8]. Isolates were preserved in Trypticase soy broth media (TSB) containing 20 % glycerol and stored at −70°C until used [9].

Susceptibility testing

The antimicrobial susceptibility testing by KirbyBauer disk diffusion method was performed according to CLSI guidelines [10]. The antibiotics used were amikacin (30µg), ciprofloxacin (5µg), ceftazidime (30µg), piperacillintazobactam (100/10µg), imipenem (10µg), meropenem (MEM-10µg), aztreonam (30 µg), colistin (10 µg) and polymyxin-B (300 units). All the disks obtained were commercially (Hi-Media Laboratories Limited. Mumbai, India). ATCC strain of Pseudomonas aeruginosa 27853 is used as control. All isolates resistant to imipenem or meropenem or ceftazidime or any two of them were considered as probable MBL producer.

Imipenem- Ethylene diamine tetra acetic acid combined disc test: A lawn culture of test isolate was prepared. Allowed to dry for five minutes. Two imipenem (10 µg) discs, one with 0.5 M EDTA and other a plain imipenem disc, were placed on the surface of agar plates approximately 30mm apart. The plates were incubated overnight at 37oC for 16-18h. An increase in zone diameter of >7mm around the imipenem EDTA disc in comparison to imipenem disk alone indicates the production of MBL.

Modified Hodge Test (MHT): A saline suspension of a 0.5 McFarland standard of E. coli ATCC 25922 was prepared and diluted 1:10 and lawn inoculated on Muller Hinton Agar (MHA). After drying the plate for 3-10 minutes imipenem (10 µg) disk was placed at the center and 3-5 colonies of test organisms was inoculated in a straight line drawn out from the edge of the disk. A known NDM positive strain was used as Positive control and incubated overnight at 350C for 20-24h. the presence of a distorted zone of inhibition or cloverleaf type of indentation at the intersection of the test organism and E. coli. within the zone of inhibition of the IPM disk was interpreted as a positive result. [10-12].

Genotypic Detection of P. *aeruginosa*

The DNA was extracted from *P. aeruginosa* using Qiagen DNA Extraction Kit as per manufactures guidelines.

Molecular analysis

PCR for carbapenem encoding gene Isolates tested positive in the phenotypic test are subjected to conventional PCR for detection genes coding for MBLs.

Polymerase chain reaction (PCR) was carried out for detection of *bla*IMP, gene on a thermal cycler (Eppendorf, Germany) .The primer pair sequences used in this study and the PCR conditions is described in the below Table no. 1. The DNA extraction was performed and the electrophoresis unit was run where 2% agars gel was prepared with ethidium bromide. The bromophenonol blue dye was used for loading our DNA product which was then visualized in the gel documentation system.

Positive controls used in this test were SPM-1 producing *P. aeruginosa* 16 strain (provided by Prof. Patrick Nordmann), *blaIMP-1* from *Seratia marquises* (sequenced by Bioneer company), and *balVIM*. *P. aeruginosa* ATCC 27853 was used as a negative control [13].

Table No.1: The Nucleotide sequences of primers used for detection of metalo-beta lactamase genes.

Primer name		PCR Condition				
		Denaturing	Anneal	Extension	Cycles	Size(bp)
bla_{IMP-1}	5′ TGAGCAAG TTATCTGTA TTC 3′	94°C, 60 s	57 °C, 60 s	72° C, 2 min	35	740
	5′ TTAGTTGCT TGGTTTTGA TG 3′					

Results

In our study a total of 200 clinical isolates of P. aeruginosa that was cultured from the clinical samples was studied. Out of 200 clinical isolates, there was 86 (43%) was isolated from urine, 37 (18.5%) from trachea, 32 (16%) from lesion, 27 (13.5%) from blood, 18 (9%) from pus.

The Antibiotic sensitivity test was also performed of the test isolates as per the CLSI guidelines. There were 130 isolates which was found resistant to imipenem. Of the 130 imipenem resistant isolates, 108(83%) was MBL producer as determined by CDDT. All MBL producing isolates was found resistant to the examined antibiotics.

Table No. 2: The Resistance pattern of P. aeruginosa isolations

Antibiotics	R	I	S	Percentage of resistance	Percentage of sensitivity
Imipenem (10µg)	130	3	67	65%	33.50%
Meropenem (10µg)	139	2	59	69.50%	29.50%
Ceftazidime (30µg)	142	-	58	71%	29%
Carbenicillin (100µg)	148	4	48	74%	24%
Tobramicin (10µg)	144	2	54	72%	27%
Amikacin (30µg)	126	8	66	63%	33%
Ticarcillin (75µg)	148	-	52	74%	26%
Gentamicin (10µg)	140	4	56	70.50%	28%
Cefotaxime (30µg)	149	26	25	74.50%	12.50%
Ceftizoxime (30µg)	153	18	29	76.50%	14.50%

Genomic analysis

The results of amplified genes by PCR showed that 28 MBL-producing isolates contained blaIMP-1. These 28 isolates was isolated from urinary tract infection (n=15), tracheal aspirates (n=5), pus (n=2), blood culture (n=2), ear infection (n=3) and eye infection (n=1).

The DNA Extraction was performed by the Qiagen DNA kit and the DNA was isolated from the samples.

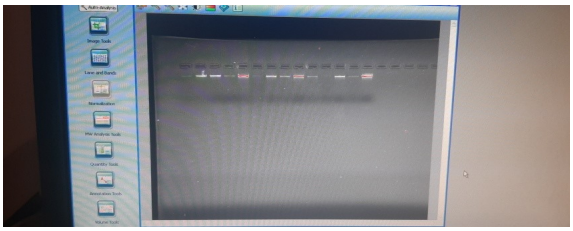

Figure 1: Photograph of DNA from P. aeruginosa isolates

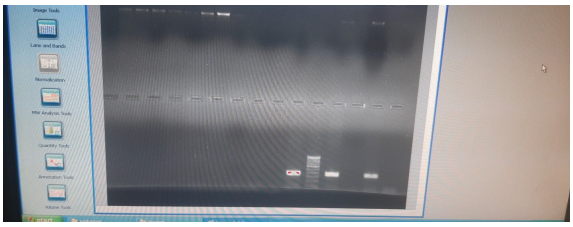

Figure 2: Photograph of amplified blaIMP gene in P. aeruginosa; the amplified DNA band size was obtained 256bp, Lane 1 and L 3 is the sample positive for blaIMP; L2 corresponding to 100bp ladder used; L4 corresponds to the Negative control and L5 corresponds to the Positive control

Discussion

The carbapenems such as imipenem generally represents main resources for treatment of infections caused by the Gram-negative opportunistic bacteria such as P. aeruginosa strains [14]. Pseudomonas aeruginosa is an opportunistic pathogen causing serious diseases in immunocompromised patients. In recent years, nosocomial infections with MBL producing strains of this organism have emerged. The detection of P. aeruginosa carrying MBLs has become a must to detect because of their role in serious infections like septicemia and pneumonia and resulted to mortality and morbidity in different parts of the world [15, 16].

In our study a total of 200 clinical samples were included for study. Theses bacteria had been isolated from different clinical specimens such as urine, wound, blood, trachea and other clinical specimens. The susceptibility pattern of these isolates was tested and the impanel resistant isolates were assessed for MBL using IMP-EDTA disks and molecular analysis targeting blaIMP. MBLs are a group of β-lactamase enzymes which need one or two zinc in their active site to cleave

the amide bond of the β-lactam ring to inactive β-lactam antibiotics [17]. In our study the rate of resistant to imipenem was 65% and most of them were positive for production of MBL. This study was similar to the study performed by the other author where the rate of resistant was found between 11 to 61% [18-20], but in contract with the study by Plotto et al [21] where it showed that 54/56 (96.4%) of isolates was resistant to imipenem. They also showed that 17 /56 (30.3%) of imipenem-resistant strains was positive for production of MBL (4) which is less than rate we found in the current study.

In our study out of 200 clinical samples tested for blaIMP-1, the results of amplified genes by PCR showed that 28 MBL-producing isolates contained blaIMP-1. This study was similar to the study performed by the other author where the blaIMP gene was detected [22]. There was another study also performed by A. Farajzadeh et al., where the blaIMP-1 gene was isolated. There was other studies which contradict our results where the blaIMP gene was not detected. A study by Khosravi and Milhani performed on burn patient in Ahvaz stated that 8(19.51%) isolates was positive for blavim genes whereas none of the isolates was positive for blaimp genes [18]. There was another study by Bahar et al., where none of the isolates dertected blaIMP gene.

Resistance of P. aeruginosa isolates to imipenem due to MBL enzymes is increasing in the Kanpur Distt. Thus, rapid detection of MBL producing strains and followed by appropriate treatment is necessary...

Conclusion

MBL detection remains a controversial issue, and clinical laboratories are in need of a simple and direct method to recognize such resistance in gram-negative bacteria to improve disease management. By using new methods for rapid identification of MBL positive bacteria in the patients, we could prevent spreading of metallo-beta lactamase strains to other patients.

There should be strict management programs for reducing antibiotic overuse, adherence to hand hygiene, early detection of MBLs producer isolates, education and training in antibiotic prescribing, environmental cleaning, contact precautions, and active surveillance programs for prevention and spread of these strains.

References

1. Singh SP, Shariff M, Barua T, Thukral SS. Comparative evaluation of phenotypic tests for identifi cation of Metallo beta-lactamases producing clinical isolates of Pseudomonas aeruginosa. Indian J Med Res. 2009; 129:713–5.
2. Livermore DM, Woodford N. Carbapenemases: a problem in waiting? Curr Opin Microbiol. 2000; 3(5):489–95.
3. Bradley JS, Garau J, Lode H, Rolston KV, Wilson SE, Quinn JP. Carbapenems in clinical practice: a guide to their use in serious infection. Int J Antimicrob Agents. 1999; 11(2):93–100.
4. Polotto M, Casella T, Oliveira MG, Rubio FG, Nogueira ML, Almeida MTG, et al. Detection of Pseudomonas aeruginosa harboring blaCTX-M-2, blaGES-1 and blaGES-5, blaIMP-1 and blaSPM-1 causing infections in Brazilian tertiary-care hospital. BMC Infect Dis 2012; 12: 176.
5. Sadeghi A, Rahimi B, Shojapour M. Molecular detection of metallo-β-lactamase genes blaVIM-1, blaVIM-2, blaIMP-1, blaIMP-2 and blaSPM-1 in Pseudomonas aeruginosa isolated from hospitalized patients in Markazi province by Duplex-PCR. Afr J Microbiol Res 2012; 6: 2965– 2969.
6. Liakopoulos A, Mavroidi A, Katsifas E, Theodosiou A, Karagouni AD, Miriagou V, et al. Carbapenemase-producing Pseudomonas aeruginosa from central Greece: molecular epidemiology and genetic analysis of class I integrons.BMC Infect Dis 2013;13 (505): 1– 7.
7. Rizek C, Fu L, Dos Santos LC, Leite G, Ramos J, Rossi F, Guimaraes T, Levin AS, Figueiredo Costa S. Characterization of carbapenem-resistant Pseudomonas aeruginosa clinical isolates, carrying multiple genes coding for this antibiotic resistance. Ann Clin Microbiol 2014; 13: 1– 5.
8. Livermore DM, Woodford N. Carbapenemases: a problem in waiting? Curr Opin Microbiol. 2000; 3(5):489–95.
9. Poirel L, Nordmann P. Acquired Carbapenem-Hydrolyzing BetaLactamases and their Genetic Support. Curr Pharm Biotechnol. 2002; 3(2):117–27.
10. Performance Standards for Antimicrobial Susceptibility Testing: Twentieth Informational Supplement M100-S20-U. Wayne, PA: CLSI; 2020.
11. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. Imipenem-EDTA Disk Method for Differentiation of Metallo- - Lactamase-Producing Clinical Isolates of Pseudomonas spp. and Acinetobacter spp. J Clin Microbiol. 2002; 40(10):3798–3801.
12. Lee K, Lim YS, Yong D, Yum JH, Chong Y. Evaluation of the Hodge Test and the Imipenem-EDTA Double-Disk Synergy Test for Differentiating Metallo- - Lactamase-Producing Isolates of Pseudomonas spp. and Acinetobacter spp. J Clin Microbiol. 2003; 41(10):4623–9.
13. Sarhangi M, Motamedifar M, Sarvari J. Dissemination of Pseudomonas aeruginosa Producing blaIMP1, blaVIM2, blaSIM1, blaSPM1 in Shiraz, Iran. Jundishapur J Microbiol 2013; 6: e6920.
14. Ryoo Nam Hee, Ha Jung Sook, Jeon Dong Seok, Kim Jae Ryong. Prevalence of Metallo-β-lactamases in Imipenem-non-susceptible Pseudomonas aeruginosa and Acinetobacter baumannii. Korean J Clin Microbiol. 2010; 13(4):169-72.
15. Shahcheraghi F, Nikbin VS, Feizabadi MM. Identification and genetic characterization of metallo-beta-lactamase-producing strains of Pseudomonas aeruginosa in Tehran, Iran. New Microbiol. 2010; 33(3):243-8.
16. Libisch B, Balogh B, Fuzi M. Identification of two multidrugresistant Pseudomonas aeruginosa clonal lineages with a countrywide distribution in Hungary. Curr Microbiol. 2009; 58(2):111-6.
17. Bebrone C, Bogaerts P, Delbrück H, Bennink S, Kupper MB, De Castro R, et al. GES-18, a new carbapenem-hydrolyzing GES-type beta-lactamase from Pseudomonas aeruginosa that contains Ile80 and ser170 residues.Antimicrob Agents Chemother 2013; 57: 396–401

18. Khosravi AD, Mihani F. Detection of metallo-beta-lactamase- producing Pseudomonas aeruginosa strains isolated from burn patients in Ahwaz, Iran. Diagn Microbiol Infect Dis. 2008; 60 (1):125-5
19. Shahcheraghi F, Nikbin VS, Feizabadi MM. Identification and genetic characterization of metallo-beta-lactamase-producing strains of Pseudomonas aeruginosa in Tehran, Iran. New Microbiol. 2010; 33 (3) : 243 -8.2
20. Shahcheraghi FN, Shooraj VS, Shafiei F, Pejouhandeh M. Investigation of blaIMP-1, blaVIM-1 and bla SPM-1MBL genes among clinical strains of Pseudomonas aeruginosa isolated from Imam Khomeini Hospital, Pajoohandeh J. 2009;14(2).
21. Polotto M, Casella T, Oliveira MG, Rubio FG, Nogueira ML, Almeida MTG, et al. Detection of Pseudomonas aeruginosa harboring blaCTX-M-2, blaGES-1 and blaGES- 5, blaIMP-1 and blaSPM-1 causing infections in Brazilian tertiary-care hospital. BMC Infect Dis. 2012; 12:176.
22. Fallah F, Shams Borhan R, Hashemi A. Detection of bla(IMP) and bla(VIM) metallo-β- lactamases genes among Pseudomonas aeruginosa strains. Int J Burn Trauma 2013; 3:122-124.