Sir Salimullah Medical College Journal

Sir Salimullah Med Coll J 2022; 30: 35-39

Original Article

Detection of Metallo β-Lactamases in Clinical Isolates of *Pseudomonas aeruginosa* in a Tertiary Care Hospital

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Article information
Received: 01-11-2021
Accepted: 05-12-2021

Cite this article:
Farzana A, Shamsuzzama SM, Akhter S, Begam M, Jahan H. Detection of Metallo β-Lactamases in Clinical Isolates of *Pseudomonas aeruginosa* in a Tertiary Care Hospital. Sir Salimullah Med Coll J 2022; 30: 35-39

Key words:
P. aeruginosa, MDR, *bla* VIM, *bla* NDM-1, MBL, PCR.

Abstract

Background: *Pseudomonas aeruginosa are among the major nosocomial pathogens that demonstrate all known enzymatic and mutational mechanism of bacterial resistance. They are able to acquire drug resistant determinants by horizontal transfer of mobile genetic elements coding for class B carbapenemases, called metallo β lactamases which hydrolyze all β lactams except aztreonam. MBLs like bla VIM has been reported among isolates of Enterobacteriaceae family, P.aeruginosa and other gram negative nonfermenters. On the other hand NDM-1 was first identified in Klebsiella Pneumoniae. Its spread among Pseudomonaceae implies possibility of numerous New NDM-1 cases to be detected in near future.*

Objective: *The study aimed to isolate VIM and NDM-1 producing* P. aeruginosa *from burn wound and to see their susceptibility pattern.*

Materials and methods: *P. aeruginosa was isolated by culturing on Macconkey agar media both on 37^0 C and 42^0 C and biochemical test. For gene detection organisms were stored at -70^0C, bla VIM and bla NDM-1 was detected by PCR. Susceptibility pattern of organisms were done by Kirby Bauer Disc Diffusion Method.*

Result: *Among the 98 isolated* P. aeruginosa *21(47.7%) were VIM producers and 6 (13.6%) NDM-1 producers. VIM producers were 80.9% resistant to imipenem and NDM-1 producers were 100% resistant imipenem.*

Discussion: *VIM producing P. aeruginosa was isolated from burn patient in accordance with other study, NDM-1 producing P. aeruginosa is emerging that are more resistant to conventional antibiotcs even to the last resort drug imipenem.*

Conclusion: *Imipenem resistence is increasing among P. aeruginosa as a result of acquiring MBL genes. Risk factors for acquiring resistance is increased carbapenem use, longer hospital stay, ICU admission, being on total parenteral nutrition, using catheter and tubes. In this study patient in whom imipenem resistant isolates were identified had hospital stay more than 7 days*

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that is ubiquitous in nature. It is one of the most important causes of nosocomial infection specially in burn patients, which is not only due to high prevalence and severity but also because of its innate and acquired resistance to antibacterial drugs[1]. Current isolates of *P.aeruginosa* are often multidrug resistant. It possesses an intrinsic resistance to many antibiotics and has an ability to develop resistance through mutation in different chromosomal loci or through the horizontal

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acquisition of resistant genes which are carried on plasmid, transposons or integrons[2]. Carbapenems (imipenem, meropenem) are classified as β-lactam antibiotics. The introduction of carbapenems into clinical practice provided a great advance in treatment of serious bacterial infections caused by beta lactamase producing bacteria. Due to broad spectrum of activities and stability to hydrolysis by most beta- lactamases (ESBLs and AmpC β-lactamases) carbapenems have become the drug of choice for treatment of infections caused by penicillin or cephalosporin-resistant Gram-negative bacilli, especially ESBL producing gram-negative infections[3]. Carbapenem resistance has been frequently observed in nonfermenting bacilli, such as *P. aeruginosa* and *Acinetobacter spp*. Resistance to carbapenem is due to decreased outer membrane permeability, increased efflux system, alteration of penicillin binding proteins and carbapenem hydrolyzing enzymes-carbapenemase. These carbapenemases are class B metallo-β-lactamases (IMP,VIM,SPM, NDM) or class D oxacillinases (OXA 23 to OXA 27) or class A clavulanic acid inhibitory enzymes (SME, NMC, IMI, KPC)[4]. Metallo-β-lactamases are able to hydrolyze all β-lactams except monobactam[5]. Class B metallo-β-lactamas requires a divalent cations of zinc as cofactors for enzyme activity. The IMP and VIM genes responsible for MBL production are horizontally transferable via plasmids and can rapidly spread to other bacteria[6]. VIM and IMPs are the most frequent MBLs acquired by gram negative bacilli[7]. MBL producing *P.aeruginosa* was first reported in Japan in 1991[8] since then its incidence has been reported from various parts of the world. VIM-1 was first reported in *P. aeruginosa* in Italy in 1997[9], followed by reports of VIM-2 in France and Greece. NDM-1 represents the recent type of mobile metallo β-lactamase to appear but is behaving differently in terms of rapidity of its spread and scope of organism in which it is found[10]. NDM was predominantly found in *Enterobacteriaceae* and mostly in *Klebsiella. pneuminiae* and *Escherecia.coli* isolates. Cases among gram negative non fermenters such as *Acinetobacter spp* that produce NDM have also been reported. More recent publications report cases also among *Pseudomanas spp*. that produce NDM. The ability to spread not only among Enterobacteriaceae but also among other bacterial families like *Pseudomanaceae* implies the possibility for numerous new NDM-1 cases to be detected in the near future. Because of its ability to spread, carbapenem resistance has become a serious concern[11]. Immediate detection of MBL producing *P. aeruginosa* is important to prevent the spread of organism within and between hospitals and to accurately treat infections caused by these organisms. To reduce healthcare cost and prolonged hospital stay, a regular monitoring of incidence β-lactamase producing organisms has become a need of time[12].

Materials and Methods

Bacterial Isolates:

This investigation was approved by research review committee (RRT) and ethical review committee (ERC) of Dhaka Medical College. During the period from July 2011 to December 2012, a total of 98 non duplicate isolates of *P. aeruginosa* were collected from burn unit of Dhaka Medical College Hospital , Dhaka, Bangladesh. Isolates from burn wound were cultured on blood agar and MacConkey agar media and stored at -70^{0}C and studied after being sub- cultered on MacConkey agar media. Identification of Pseudomonas aeruginosa was done if were oxidase positive, a triple sugar iron (TSI) agar reaction of alkaline over no change, motile, indole and urease negative in motility-indole-urea (MIU) agar media, citrate utilized in simmons citrate agar media and grew at both 37^{0}C and 42^{0}C. Additional bacterial characteristics including its Gram stain, colony morphology, hemolytic criteria and pigment production were also used to identify the species.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing for all the collected samples was done by the Kirby-Bauer method using Mueller Hinton agar plates as recommended by Clinical and Laboratory Standards Institute (CLSI; 2010)[13]. The following antibiotic were tested: imipenem (10 µg), meropenem (10µg), ceftazidime (30µg), cefepime (30µg), ceftriaxone (30µg), gentamycin (10µg), amikacin (30µg), ciprofloxacin (5 µg), colistin (10µg) (Oxoid ltd. UK). When the isolate was resistant to three or more classes of antipseudomonal agents (i.e. penicillin / cephalosporins, carbapenems, aminoglycosides and fluoroquinolones) that isolate was considered as multidrug resistant (MDR). If more than one agent

within a class were used, the isolate was considered resistant to all of the agents[14]. *P. aeruginosa* which displayed resistance to imipemem or meropenem by disc diffusion method (zone size for imipenem and meropenem ≤15mm) were selected for PCR for detection of MBL gene since MBL was responsible for carbapenem resistance. The minimum inhibitory concentration (MIC) of imipenem was obtained by agar dilution method. Susceptibility and MICs breakpoint was defined according to CLSI[13]. Carbapenem resistance was determined based on MIC points. *P. aeruginosa* ATCC 27853 was used as control strain in all susceptibility assays.

Detection of MBLs by PCR

Polymerase chain reaction (PCR) analysis was performed for *bla* VIM and bla NDM-1 genes (VIM-F: 5'CCGATGGTGTTTGGTCGCAT 3', VIM-R: 5'GAATGCGCAGCACCAGGAT3' with product size of 391 bp and annealing temperature at 62.4^{0}C) (NDM-1 F:5'ACCGCCTGGACCGATGACCA3', NDM-1R: 5'GCCAAAGTTGGGCGCGGTTG3'with product size 200bp and annealing temperature 63^{0}C). DNA was extracted using the boiling method. Three hundred micro liter of sterile distilled water was added into eppendorf tubes having bacterial pellet and vortexed until mixed. Mixture was heated at 100^{0}C for 10 minutes in a heat block. After heating, immediately the eppendorf tubes were placed on ice for 5 minutes and then centrifuged at 20,000 g at 4^{0}C for 6 minutes. Supernatant was taken into another eppendorf tube by micropipette and was used for PCR. The primers were mixed with Tris-EDTA (TE) buffer according to manufacturer's instruction. For each sample, a total of 25µl of mixture was prepared by mixing of 12.5µl of master mix (mixture of dNTP, Taq polymerase $MgCl_2$ and PCR buffer), 2 µl foreward primer, 2µl reverse primer (Promega Corporation, USA), 2 µl of DNA template and 6.5 µl of sterile distilled water in a PCR tube. After a brief vortex, the tubes were centrifuged in a micro centrifuge for a few seconds. DNA was amplified in a Mastercycler Eppendorf (Eppendorf, Germany) under the following conditions: initial denaturation at 95^{0}C for 10 minutes, then 30 cycles of denaturation at 95^{0}C for one minute, annealing for 45 seconds, extension at 72^{0}C for one minute and 30 seconds and final extension at 72^{0}C for 10 minutes. Amplicons were kept at 4^{0}C for gel electrophoresis. For gel electrophoresis, 1µl of loading dye was placed on parafilm. 5 µl amplicon was mixed with loading dye on parafilm and the mixture was loaded into well of agarose gel. 2 µl of DNA ladder was mixed with 1 µl of loading dye and loaded into well of agarose gel and placed on electrophoresis tank having 1X Tris -Borate-EDTA (TBE) buffer. Gel electrophoresis was done at 100 voltages for 35 minutes. Gel was stained with staining solution of ethidium bromide and destained by keeping in sterile distilled water for 15 minutes. Gels were visualized and photographed under ultraviolet illumunation. *P.aeruginosa* ATCC27853 reference strain was used as the *bla* MBL gene's negative control strain.

Statistical Analysis

Data were analyzed by using Microsoft Excel (2007) software (Microsoft, Redmond, WA, USA).

Result

A total of 98 (44.95%) *P. aeruginosa* were isolated from 222 samples of them 75 (76.53%) were resistant to 3rd generation cephalosporins (ceftazidime 30µg), 35 (35.72) were resistant to imipenem, 33(33.68) were resistant to meropenem zone size diameter 13mm for imipenem and meropenem. Among them 10 (43.48) were ESBL producer as detected by double disc diffusion test. Out of 44 imipenem and meropenem resistant *P. aeruginosa*, Twenty seven (61.36%) MBL producers were detected by PCR of which 21(47.72%) were positive for *bla*VIM, 6(13.64%) for *bla*NDM-1 and 17 (38.64%) had no MBL gene. On the basis of MIC 4 (16%) out of 25 of these imipenem resistant *P.aeruginosa* was susceptible to imipenem thus indicating that resistant in disc diffusion could show susceptibility by MIC. All these MBL producing isolates were tested for their susceptibility to colistin by disc diffusion method.

Table I

Distribution of bla MBLs among Carbapenen resistant strains of P aeruginosa (n=44)

MBL genes	Number (n)	Percent (%)
bla VIM	21	47.72%
bla NDM-1	6	13.64%
Absence of examined genes	17	38.64%

Table II

Resistance pattern of MBL producing P aeruginosa

MBL gene	Disc Diffusion Method								
	IMP	MEM	CRO	CAZ	FEP	AK	CN	CIP	CT
VIM (n=21)	17 (81.0%)	20 (95.2%)	21 (100%)	21 (100%)	21 (100%)	21 (100%)	21 (100%)	21 (100%)	2 (9.5%)
NDM-1 (n=6)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	1 (16.7)

IMP: Imipenem, MEM:Meropenem, CRO:Ceftriaxone, CAZ:Ceftazidime, FEP:Cefepime, AK:Amikacin, CN:Gentamicim, CIP:Ciprofloxacin, CT:Colisti

Discussion

Antibiotic resistance among gram negative pathogens such as *P. aeruginosa* is one of the major problems in treating hospitalized patients. Patients in intensive care unit, oncology departments, burn units and surgery wards frequently show multi resistant isolates which contributes to high morbidity and mortality[15]. Imipenem and meropenem are used for treatment of nosocomial infections but increasing resistance to these antibiotics has limited their effectiveness. In this study, *P. aeruginosa* was 64.28% sensitive to imipenem and 66.32% sensitive to meropenem (Table I). Saha[16] et al (2011) found 98.72% of *P. aeruginosa* sensitive to imipenem. This increased trend of resistance may be due to selective pressure on carbapenems as a result of increased use of carbapenem, increase in carbapenemase production and intrinsic resistance of *P. aeruginosa*. In Japan, rate of resistance to carbapenem increased from 19.3% in 1998 to 38% in 2002[17]. The result of this study indicates that *P.aeruginosa* was slightly less resistant to meropenem (33.68%) than imipenem (35.72%). Meropenem need mutation of two genes, loss of oprD and up regulation of mexAB efflux pump for intrinsic drug resistance which is hard achieve[18]. Present study identified 27 (61.36%) MBL producers by PCR out of 44 carbapenem resistant *P. aeruginosa* of which 21(47.72%) were VIM producer, 6(13.64%) were NDM-1 producer (Table-2). Previous study in Tehran, Iran revealed 72% VIM producers by Double Disc Synergy Test among imipenem resistant P.aeruginosa[19.] Farzana *et al*[20] found 87.50% MBL producers among *P. aeruginosa* which is higher than this study as they detected IMP-1, IMP-2, VIM-1, VIM-2 in that study. Johan *et al* [21] detected 43% *bla*VIM among *P.aeruginosa* which is similar to the study. 18% VIM-1 producing isolates were identified by Ramazan *et al*[22] which is lower than our result. The findings of present study revealed that VIM producing *P. aeruginosa* are prevalent in Bangladesh. A study in Bangladesh demonstrated 3.5% NDM-1 producers among the imipenem resistant organisms and no imipenem resistant *Pseudomanas* harbored NDM-1[23]. The ability of NDM-1 to spread not only among Enterobacteriaceae but also among other bacterial families, like *Pseudomonaceae* implies the possibility for numerous new NDM-1 cases to be detected in the near future. Tolemen *et al*[24] reported rapid emergence of new NDM-1 cases might be expected by following the chronology of VIM-1 spreading.

Acknowledgements

Department of Microbiology, Dhaka Medical College, Dhaka provided financial and laboratory support to perform this study. We thank the burn unit of Dhaka Medical College Hospital for providing samples.

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