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DETECTION OF METALLO-BETA-LACTAMASE GENES IN CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA* IN GENERAL HOSPITAL, BIDA

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen and a major cause of nosocomial infections especially in immunocompromised patients due to its extraordinary capability to acquire resistance to different antibiotics. Acquired metallo-beta–lactamase (MBLs) in *Pseusomonas aeruginosa* is worrisome and poses a great threat in terms of treatment and infection control. This study determined the antibiotic susceptibility and the prevalence of MBL genes in carbapenem-resistant *P.aeruginosa* isolated from different

Introduction

Background

Pseudomonas aeruginosa, a flagship member of *Pseudomonas* species is an aerobic Gram-negative rod-shaped opportunistic bacteria known to cause diseases in plants, animals and humans.[1] This bacillus has emerged as a major nosocomial pathogen, responsible for about 10% of all nosocomial infections especially in

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clinical samples in general hospital, Bida, Niger State, Nigeria.Twenty five non-duplicate *P. aeruginosa* isolated from various clinical samples from general hospital Bida were included in this study Antibiotic susceptibility testing was done using Kirby Bauer disk diffusion method, while the phenotypic idetection of MBL-producing strains was carried out using the combined disc test. The MBL-encoding genes were detected by polymerase chain reaction (PCR). Out of the 25 *P. aeruginosa* strains, 7(28%) were resistant to imipenem. After carrying out the phenotypic detection test, 6 strains were identified as MBL-producers but only 1 (4%) harbored the blaVIM-1 gene. No other MBL gene was detected. In conclusion, this study established the presence of MBL genes among *P. aeruginosa* strains in the study area. Therefore, there is need for stringent and regular surveillance in the use of carbapenems and the establishment of approriate control measures to curtail spread.

Keywords: Bida, *Pseudomonas aeruginosa*, carbapenem-resistance, metallo-beta-lactamse, polymerase chain reaction

Immunocompromised patients due to its extraordinary capability to develop additional in-vivo resistance to different antibiotics.[1, 2]

Antipseudomonal beta-lactam antibiotics such as penicillins, cephalosporins, carbapenems, monobactams and selected fluoroquinolones are used for treating pseudomonal infections.[3] Of all these, the carbapenems (imipenem and meropenem) used to be reserved as the last resort, especially in the face of MDR Gram-negative bacteria. However, carbapenem resistance, especially in *P. aeruginosa* is on the rise. *P. aeruginosa* exhibits high level of intrinsic and acquired resistance to many antibiotics, thus making treatment of infections caused by this bacteruim challenging. Decreased outer membrane permeability, activity of the efflux pumps and enzyme production, especially β-lactamase production are resistance mechanisms exhibited by *P. aeruginosa*.[4] The emergence of strains of *P. aeruginosa* with acquired metallo-beta-lactamase (MBLs) is worrisome because it poses a serious challenge

clinically both in terms of treatment and infection control.[5] Metallo-β-lactamase enzymes can efficiently hydrolyze all other β-lactam antibiotics including penicillin, cephalosporins, cephamycins with the exception of monobactams.[4,6] The mortality attributable to infections caused by MBL-producing *P. aeruginosa* is estimated to range from 70% to 90%.[5,7] Metallo-beta-lactamases belongs to the Ambler Class B of the structural classification of β-lactamases and require divalent cations, usually zinc,as a cofactor for enzyme activity.[8] They are inhibited by metal chelators such as ethylene diamine tetra acetic acid (EDTA) but not affected by therapeutic β-lactamase inhibitors like sulbactams or tazobactams. The types of MBL genes identified in *P. aeruginosa* include Verona integron-encoded metallo-β-lactamase (VIM), imipenemase (IMP), Seoul imipenemase (SIM), Germany imipenemase (GIM), São Paulo metallo-β-lactamase (SPM), New Delhi metallo-β-lactamase (NDM) types, Florence imipenemase (FIM-1), Australian imipenamase (AIM), Dutch imipenemase (DIM) and Hamburg MBL (HMB). Of these, VIM and IMP are the most prevalent types of acquired MBLs.

Pseudomonas aeruginosa producing metallo-β-lactamases (MBLs) was first reported from Japan in 1991[9] and since then has been reported in various parts of the world including China, India, Taiwan, Singapore, Italy, Australia, Germany, Spain, USA, Mexico, Korea, Netherlands, France, Greece and Bulgaria. In Nigeria, a number of studies have reported the detection of MBL among clinical isolates of *P. aeruginosa.* [5,10,11,12,13,14,15,16,17,18,19] In Niger State, there is pausity of data on MBL-induced resistance, as neither the prevalence rate of MBL-producing strains of *P. aeruginosa* nor the MBL gene types in *P. aeruginosa* has previously been reported. Therefore, this study aimed to identify the antibiotic susceptibility, phenotypic detection of MBL and determine the prevalence of MBL genes in carbapenem-resistant *P. aeruginosa* as a way of generating local data for planning antibiotic management and infection control.

Materials and Methods

Bacterial Isolates

A total of 25 non-duplicate *P. aeruginosa* isolated from various clinical samples from general hospital Bida, Niger State, Nigeria between December, 2021 and November, 2022 were included in this study. The isolates were identified using microbiological and biochemical methods. Demographic data and other relevant details of patients from whom *P. aeruginosa* was isolated were collected from the hospital medical record department. Ethical clearance was obtained from the Ethics and Research Committee of the General Hospital Bida, Niger State.

Antibiotic Susceptibility Testing

All isolates of *P. aeruginosa* were subjected to antibiotic susceptibility testing using the Kirby–Bauer disc diffusion method on Mueller-Hinton agar (MHA) plates. The following antibiotic were tested: imipenem (IPM:10µg), meropenem (MEM:10 µg), ceftazidime (CAZ:30µg), cefotaxime (CTX:30µg), cefepime (CEF:30µg), gentamicin (GEN:10µg), amoxicillin (AMX:30µg), piperacillin/tazobactam (TZP:100/10µg), colistin (CST:10µg), aztreonam (ATN:30µg) norfloxacin (NFX:10 µg) and ciprofloxacin (CIP:5 µg) (Mastdiscs, Britain). The results were interpreted as per the Clinical Laboratory Standard Institute (CLSI) guidelines

Phenotypic Detection of Metallo-beta-lactamse Production

All carbapenem (imipenem and meropenem) resistant and intermediately resistant isolates were screened for metallo-β-lactamase enzymes using the combined disc test (CDT). In this method, two imipenem discs, (10µg each) were placed 20mm apart on a Mueller Hinton agar plate previously seeded with 0.5 McFarland standard of overnight bacterial isolate suspension. Ten microliter (10µl) of 0.5M EDTA was then added to one imipenem disc and the plate was incubated at 37^{o}C for 24 hours. The inhibition zone of imipenem alone and IMP+EDTA were measured. An increase of ≥7mm or more in zone diameter of EDTA

containing imipenem disc compared to imipenem alone disc was considered positive for metallo-β-lactamase enzymes production.

Detection of MBL Genes by PCR

All Carbapenem-resistant isolates that were positive for the phenotypic detection were subjected to polymerase chain reaction (PCR) for the detection of MBL genes. The genes include bla*VIM-1*, bla*VIM-2*, bla*IMP-1*, bla*IMP-2*, bla*SPM-1*, bla*NDM-1* and bla*GIM*. DNA extraction was done using a commercial genomic DNA extraction kit (GeneAll Biotechnology Co. Ltd, South Korea). The primers sequences (Inqaba biotec, South Africa) used to amplify MBL genes are presented in Table 1.

Table 1: Gene-specific Primer Sequences

Gene	Primer Sequence (5′ – 3′)	Annealing Temperature(^{0}C)
bla*VIM-1*	Forward: AGTGGTGAGTATCCGACAG Reverse: ATGAAAGTGCGTGGAGAC	58
bla*VIM-2*	Forward: ATGTTCAAACTTTTGAGTAAG Reverse: CTACTCAACGACTGAGCG	52
bla*IMP-1*	Forward: ACCGCAGCAGAGTCTTTGCC Reverse: ACAACCAGTTTTGCCTTACC	55
bla*IMP-2*	Forward: GTTTTATGTGTATGCTTCC Reverse: AGCCTGTTCCCATGTAC	51
bla*SPM-1*	Forward: GCGTTTTGTTTGTTGCTC Reverse: TTGGGGATGTGAGACTAC	52
bla*NDM-1*	Forward: GGCGGAATGGCTCATCACGA Reverse: CGCAACACAGCCTGACTTTC	56
bla*GIM-1*	Forward: TCGACACACCTTGGTCTG Reverse: AACTTCCAACTTTGCCAT	52

The PCR reaction mixture had a total voulume of 25µl , PCR reaction mixture 12.5 µL of ReadyMix™ Taq PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO, USA), 2.5 µL of the DNA (20 pg), and 0.5 µM of each primer and

nuclease-free water made up to 25 µL. A tube containing all PCR reaction mixture without the DNA template was used as negative control. PCR amplifications was performed using a thermocycler (Biorad MyCyclerTM Thermalcycler, BioRad, USA). The PCR cycling conditions was as follows: initial denaturation at 96 °C for 10 min, followed by 30 cycles of 96 °C for 1 min, specific annealing temperatures of respective primers as presented in Table 1 for 1 min, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. About 3µl of amplicon of each PCR product was electrophoresed on a 1.2% agarose gel for 1 hour at 85V, visualized and photographed under UV transilluminator (GelDoc 1000 system, BioRad, USA) with 100bp DNA ladder as molecular weight marker.

Results

Out of the 90 samples screened comprising of 41 males and 49 females respectively, 25(27.8%) were positively identified as *P. aeruginosa* with males having 10 (24.4%) against females 15(30.6%) as shown in Table 2. Various clinical samples included urine (7, 7.8%), high vaginal swab (5, 5.6%), pus (3, 3.3%), blood culture (2, 2.2%), ear swab (4, 4.4%), wound swab (3, 3.3%) and urethra swab (1, 1.1%).

Table 2: **Demographic Profile of Patients with *Pseudomonas aeruginosa* Infections**

Parameters	No.(%) of Strains
Samples (n=90)	
Gender	
Male	10 (11.1))
Female	15 (16.7))
Patient category	
Out-patient	20 (22.2)
In-patient	5 (5.6)
Sample Type	
Urine	7 (7.8)

HVS	5 (5.6)
Pus	3 (3.3)
Blood culture	2 (2.2)
Ear swab	4 (4.4)
Wound swab	3 (3.3)
Urethra swab	1 (1.1)

HVS: High vaginal swab

Antibiotic Susceptibility Testing

Of the 25 *P. aeruginosa,* majority of the isolates were found resistant to norfloxacin (23, 92%), amoicillin (22, 88%), gentamicin (15, 60%), cefotaxime (14, 56%), ceftazidime (17, 68%) and ciprofloxacin (12, 48%). Low levels of resistance were observed towards colistin (2, 8%), piperacillin/tazobactam (2, 8%), imipenem (7, 28%) and meropenem (10, 40%) as presented in Table 3.

Table 3: **Susceptibility Profile of *P. aeruginosa* to Antipseudomonal Antibiotics**

Antibiotics	Susceptible	Intermediate	Resistant
Imipenem	16 (64.0)	2 (8.0)	7 (28.0)
Meropenem	14 (56.0)	1 (4.0)	10 (40.0)
Amoxicillin	3 (12.0)	0 (0)	22 (88.0)
Piperacillin/tazobactam	23 (92.0)	0 (0)	2 (8.0)
Gentamicin	9 (39.2)	1 (4.0)	15 (60.0)
Cefotaxime	10 (40.0)	1 (4.0)	14 (56.0)
Ceftazidime	8(32.0)	0 (0)	17 (68.0)
Cefepime	14(56.0)	2 (8.0)	9 (36.0)
Norfloxacin	2 (8.0)	0 (0)	23 (92.0)
Ciprofloxacin	11 (44.0)	2 (8.0)	12 (48.0)
Aztreonam	13 (52.0)	2 (8.0)	10 (40.0)
Colistin	23 (92.0)	0 (0)	2 (8.0)

Phenotypic Detection of MBL Production

All isolates that were imipenem resistant (n=7, 28%) and intermediately resistant to imipenem (n=2; 8%) were subjected to MBL detection by the combined disc test. Of the 9 *P. aeruginosa* isolates tested, 6 (66.7%) isolates were found to be positive for MBL production. Of the different samples, 100% (3/3) of *P. aeruginosa* isolated from wound swab were found to be MBL producers, followed by *P. aeruginosa* isolated from pus (2/3, 66.7%) and the last was from urine (1/7, 14.3%). All the MBL-producers were detected in *P. aeruginosa isolated* from male (6/6, 100%). Five (83.3%) out of the 6 MBL-producing strains were susceptible colistin.

Detection of MBL Genes by Polymerase Chain Reaction

Out of the 6 phenotypically positive MBL-producing *P. aeruginosa* isolates, $bla_{\text{VIM-1}}$ gene was detected in only 1(16.7%) while the remaining 5(83.3%) did not amplify any of the tested genes (Table 4). The agarose gel electrophoresis result for the $bla_{\text{VIM-1}}$ gene is shown in Figure 1.

Table 4: **Resistance Pattern and Molecular Characteristics of MBL-producing *P. aeruginosa***

Parameters	Characterisctics					
Strain no.	3	19	51	69	88	89
Sample type	U	P	WS	WS	WS	P
Gender (M/F)	M	M	M	M	M	M
Antibiotics						
Imipenem	R	R	R	R	R	R
Meropenem	R	R	R	I	I	R
Cefepime	R	R	R	R	R	I
Cefotaxime	R	I	R	R	I	R
Ceftazidime	R	I	R	R	I	R
Norfloxacin	R	R	R	R	R	R
Ciprofloxacin	R	R	R	S	R	R

Pipacillin/Tazobactam	S	S	S	S	S	R
Amoxicillin	R	R	R	R	R	R
Gentamicin	I	R	R	R	I	R
Aztreonam	S	I	I	S	I	R
Colistin	R	S	S	S	S	S
MBL gene						*bla*$_{VIM--1}$

U=urine; P =pus; WS=wound swab; M=male; F=female; R=resistant; S=susceptuble; I=intermediate

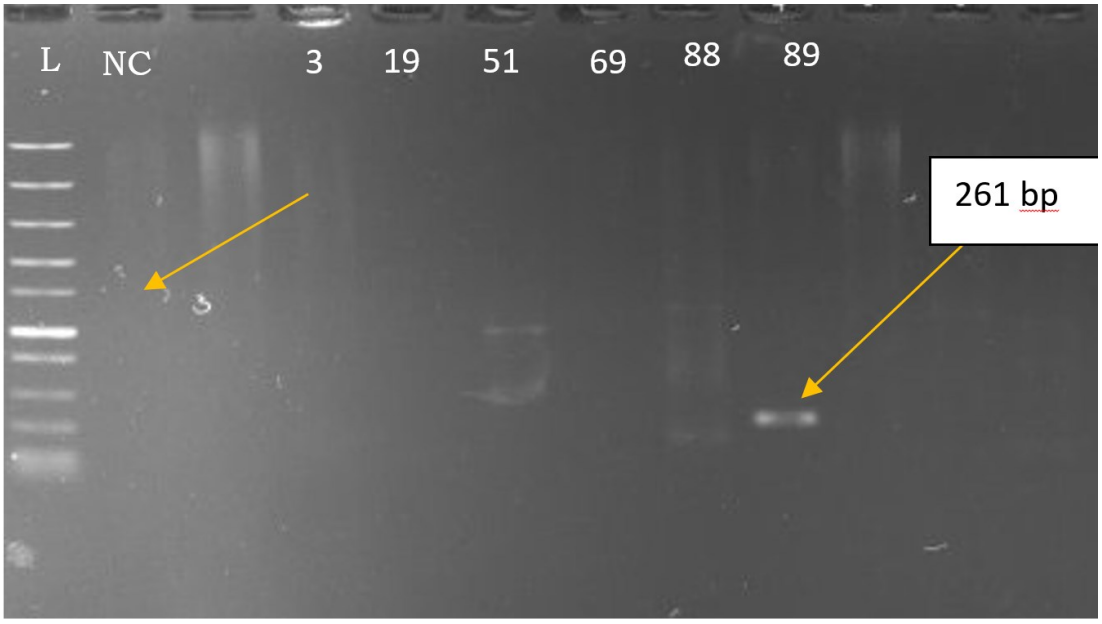

Fig 1. Gel electrophoresis of PCR products following amplification with specific primer for bal$_{VIM-1}$ gene (261 bp) From left, Lane L :DNA ladder for 1kb; Lanes **:** negative control; Lanes 3,19,51,69 and 88 are negative; Lane 89: isolate positive for bla$_{VIM-1}$

Discussion

In this study, the antibiotic susceptibility testing showed that isolates were most susceptible to colistin, piperacillin/tazobactam, imipenem, meropenem and cefepime but largely resistant to norfloxacin, amoxicillin and ceftazidime. This observation is in agreement with the prevailing

reports from Zubair and Iregbu,[5] and Adejobi *et al.*[22] that clinical isolates of *P. aeruginosa* are largely susceptible to imipenem, colistin and piperacillin/tazobactam while resistant to the third generation cephalosporin (ceftazidime). This observation may not be unconnected with the fact that carbapenems are not commonly prescribed in the study area because they are reserved as last resort drug. Invariably, there is less exposure of the bacteria to this classes of antibiotic. This reason applies to the high susceptibility of the isolates to the polymyxin B (colistin) and β-lactam + β-lactam inhibitor (piperacillin/tazobactam).

In this study, 66.7% of the isolates were found to be MBL producers. Ikpeme *et al.* [19] in Calabar, Chika *et al.* [20] in Enugu, Zubair & Iregbu[5] in The Federal Capital Territory and Onipede *et al.*[19] in Ile Ife Ogun State reported prevalences of 58.3%, 10.0%, 11.0%, 9.8% respectively. The differences in prevalence may be attributed to differences in sample size, a recent antibiotic usage or hospitalization.[4] In addition, the combined disk test is reported to be of lower sensitivity compared to the disk potentiation method used in most of the previous reports. The MBL-producing stains of *P. aeruginosa* in this study were resistant to majority of the antipseudomonal antibiotics tested but largely remained susceptible to colistin. High resistance of MBL-producing strains of *P. aeruiginosa* to a broad array of antipseudomonal antibiotics has been reported by previous studies.[4,5,22,23,24,25] In this study, majority of MBL-positive isolates were from wound samples. Some previous studies reported the association of MBL-producing *P. aeruginosa* with wound/burns.[4,22,,23,24] The physiology of a wound/burn provides a moist,warm and nitritious environment which is conducive for microbial proliferation. Some metallo-beta-lactamase genes constitute reservoirs in the environment. As some bacteria evolved, some species of Bacillus such as *Bacillus cereus* and *Bacillus anthracis* produced metalloenzymes which were used as a means of protection for them against beta-lactams produced naturally by some soil-dwelling bacteria like *Streptomyces specie* and other fungi. Therefore, the spread of carbapenemase genes is two-directional: the environmental sources may

provide the genetic sources of the enzyme while the clinial strains may spread the enzymes in the hospital environment.[26]

In this study, *bla*$_{VIM-1}$ was the only MBL gene detected in 1(16.7%) strain. The other 5 isolates were negative for all the MBL genes tested. This may indicate the possibility of the presence of other MBL genes other than those examined in this study or false-positive results which has been reportedly associated with phenotypic detection methods.[22] Of all the MBL genes known, VIM and IMP types are the most common and widespread in *P. aeruginosa*.[24] The 16.7% prevalence in our study is higher than a recent study in Ogun State by Adeleyu *et al.*[25] where the prevalence of *bla*$_{VIM}$ in clinical isolates of *P. aeruginosa* was 12.8% and the Federal Capital Territory, Abuja by Zubair & Iregbu,[5] where *bla*$_{VIM-1}$ was the only MBL gene detected in 5 out of 255 isolates of *P. aeruginosa* giving 2.5% prevalence while all other MBL genes were not detected. In another study in Yenagoa, Bayelsa State, Abdulrasheed *et al*. [16] reported *bla*$_{VIM}$ as the only MBL gene detected in 4 out of 37 *P. aeruginosa* isolates, giving a prevalence of 10.8%. The results from our study supports previous reports which suggests VIM-type as the predominant MBL gene in *P. aeruginosa*, although of low prevalence in Nigeria.

Conclusion

In conclusion, susceptibility of *P. aeruginosa* in the study area towards imipenem and meropenem is relatively high, but the detection of MBL-producing strains is worrisome as it poses a serious challenge for effective treatment and infection control. Therefore, there is need for stringent and regular surveillance in the use of carbapenems and the establishment of approriate control measures to curtail spread.

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Conflicts of Interest

There are no conflicts of interest.

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