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Molecular Characterization of Carbapenemase Encoding Genes in *Pseudomonas aeruginosa* from Tertiary Healthcare in South Eastern Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background and Objectives: In recent years, the rate of carbapenemase encoding gene in *P. aeruginosa* has increased worldwide and has become of great concern since it's significantly restricts the therapeutic options for patients in Tertiary health care. Therefore, there's a need for molecular characterization of carbapenemase encoding genes in *Pseudomonas aeruginosa* from Tertiary Healthcare in South Eastern Nigeria.
Methodology: A total of twelve (12) *Pseudomonas aeruginosa* positive culture of Urine (n=5), Wound swab (n=5), Catheter tip (n=2) were collected from Alex Ekwueme Federal University

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Hospital Teaching Hospital, Abakaliki (AE-FUTHA), Ebonyi State, South eastern Nigeria. The *Pseudomonas aeruginosa* strain confirmation was performed using VITEK 2 System and the bacteria were further screen for carbapemase encoding gene by PCR specific primer.

Results: Molecular amplification of carbapenemase encoding genes revealed that bla_{NDM} and bla_{IPM} accounted 12 (100%) across all sample source. Among the various sample sources, bla_{KPC} was found 1(8.3%) in Urine, wound swab 3(25.0%), and Catheter tip 1(8.3%), while bla_{VIM} was found 2(16.7%), 2(16.7%) and 0(0.0%) in Urine, wound swab and Catheter tip respectively. Co-expression of bla_{NDM} + bla_{IMP} accounted 5(41.6 %), 5(41.6 %) and 2(16.7 %) in Urine, wound swab and Catheter tip respectively. Co-expression of bla_{KPC} + bla_{NDM} + bla_{VIM} + bla_{IMP} + bla_{OXA} was only detected in urine 1(8.3 %).

Conclusion: The current study gives an account of the presence of carbapenemase-encoding genes in *P. aeruginosa*. The expression of carbapenemase-encoding genes may be the mainstay of phenotypic MDR. As a result, physicians, other medical professionals, researchers, and public health policymakers must be kept up to date on the spread of carbapenemase-encoding genes. In addition, strict infection prevention and control strategies, as well as antimicrobial stewardship programs, are highly desirable in admission healthcare facilities where carbapenemase-encoding genes are spreading.

Keywords: Carbapenemase-encoding; gene; Pseudomonas aeruginosa.

1. INTRODUCTION

Pseudomonas aeruginosa is an opportunistic and nosocomial pathogen that colonizes people with cystic fibrosis and is a common etiologic agent of bacteremia, urinary tract infections, wound infections, and colonizes people with cystic fibrosis, among other infections. Recently, it has been described as a pathogen that co-infects patients with COVID-19 [1,2]. Despite the advances made through the introduction of anti-pseudomonal drugs, *Pseudomonas aeruginosa* infections remain a big threat in patients due to its resistance to antibiotics. Most drugs have lost their efficacy against *Pseudomonas aeruginosa* and the susceptibility patterns change with time and regions in the world. The increase in antibiotic resistance by *Pseudomonas aeruginosa* has been observed in several epidemiological studies carried out all over the world [3].

Currently the major threat of antibiotic-resistant bacteria is from MDR Gram-negative organisms, particularly those which have developed resistance to carbapenem. Along with carbapenem-resistant *Pseudomonas aeruginosa* (CRPA), are among the top tier of the WHO list of antibiotic-resistant “priority pathogens” that pose the greatest threat to human health [4,5]. Among the clinically important bacteria that harbor plasmidic genes encoding a wide variety of carbapenemases, isolates of sequence type (ST) 258, ST11, and ST147 Enterobacterales (mainly producing *Klebsiella pneumoniae* carbapenemase [KPC], metallo-b-lactamase [MBL, especially New Delhi MBL {NDM}], and oxacillinase [OXA], etc.), ST111 and ST235 *Pseudomonas aeruginosa* (mainly producing NDM, imipenemase [IMP], Verona integron encoded MBL [VIM], and OXA, etc.) [6]. *P. aeruginosa* isolates have been reported to contain a wide variety of carbapenemases encoding gene globally. For example, in Latin America, this includes KPC, IMP, VIM, NDM [7]. In the Arabian Peninsula, carbapenemases in *P. aeruginosa* include VIM, IMP, and NDM [7]. In the United States, carbapenemases in *P. aeruginosa* include KPC, NDM, VIM, and IMP [8,9]. In another study, the pooled prevalence of among the clinical specimens in Africa OXA and VIM were the most prevailing carbapenemase among *P. aeruginosa* [10]. In recent years, the rate of carbapenemase encoding gene in *P. aeruginosa* has increased worldwide and has become of great concern since it significantly restricts the therapeutic options for patients in health care [11]. Notably, increase in carbapenemase encoding gene in clinically important *P. aeruginosa* has gradually worsened in hospitalized in patients [6,12]. The risk of colonization with carbapenamase encoding *P. aeruginosa* isolates harboring bla_{VIM}, bla_{KPC}, $bla_{IMP,}$ bla_{NDM} was also reported to apparently increase with the length of hospital stay [7,13].The emergence and rapid spread of carbapenemase encoding gene is a global concern as infections with these resistant bacteria are a matter of national and international concern as they are an emerging cause of Hospital Acquired Infections (HAIs) that pose a significant threat to public health and responsible for hospital outbreaks worldwide, thus, managing infections caused by them poses a substantial

challenge in clinical practice. Hence a robust and feasible examination of carbapenamase encoding gene in *P. aeruginosa* will serve as a guide regarding their clinical management, including prevention and treatment.

2. MATERIALS AND METHODS

2.1 Identification of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa positive culture of Urine (n=5), Wound swab (n=5) and Catheter tip (n=2) were collected from Alex Ekwueme Federal University Hospital Teaching Hospital, Abakaliki (AE-FUTHA), Ebonyi State, South eastern Nigeria. The *Pseudomonas aeruginosa* strain confirmation was performed using VITEK 2 System (bioMerieux, France) [14,15].

2.2 Molecular Characterization for Carbapamemase Encoding Genes Using Polymerase Chain Reaction (PCR)

2.2.1 Genomic DNA extraction

Genomic DNA Extraction of *Pseudomonas aeruginosa* was performed using ZR Bacterial DNA Miniprep™ (Manufactured by Zymo research, cat number: D6005) kit according to the manufacturer's protocol. Exactly 2 ml of bacterial cells broth was added to a ZR Bashing TM Lysis tube and 750 µg lysis solutions was added to it. This was secured in a bead fitted with 2 ml tube holder assembly and processed at a maximum speed for 5 minutes. The ZR Bashing Bead Tm lysis tube was centrifuged at > 10,000 x g for 1 minute. Up to 400 µg of supernatant was transferred to a Zygomo-Spin TM IV Filter in a collection tube and centrifuged at 7,000 x g for I minute. Exactly 1,200 µg of bacterial DNA Binding Buffer was added to the filtrate in the collection tube. Exactly 800 ul of the mixture from step 5 was transferred to a Zygomo-spin TM IIC Column in a collection tube and centrifuged at 10,000 x g for 1 minute. The flow through from the collection tube was discarded. Then 200 ul DNA Pre-washed buffer was added to the Zymo-Spin TM IIC Column in a new tube collection and centrifuged at 10,000 x g for 1 minute. About 200 µg of Bacterial DNA Buffer was added to the Zymo-Spin TM IIC Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin TM IIC Column was transferred to a clean 1.5 ml micro-centrifuge tube and 100 µl (35 µlminimum) DNA was added directly to the column matrix. Centrifuge was done at 10,000 x g for 30 seconds to elute DNA [16].

2.2.2 Electrophoresis for DNA and PCR

Exactly 1 g of agarose was measured (for DNA) and 2 g of agarose for PCR products. Agarose powder was mixed with 100 ml 1xTAE in a microwave flask. This was microwaved for 1-3 min until agarose iscompletely dissolved. The agarose solution was allowed to cool to about 50^{o}C. Then 10 µg EZ vision DNA stain was added. The agarose was poured into a gel tray with the well comb in place.The newly poured gel was placed at 4^{o}C for 10-15 mins until it has completely solidified [16].

2.2.3 Loading samples and running an agarose Gel

A loading Buffer was added to each DNA sample of PCR products. Once it got solidified the agarose gel was placed into the gel box (electrophoresis unit). The gel box was filled with 1xTAE buffer until the gel was covered. A molecular weight ladder was carefully loaded into the first lane of gel and the samples were carefully loaded into the additional wells of gel. The gel was run at 80-150 V for about 1-1.5 h. The power was turned off, and the electrodes disconnected from the power source and then the gel was carefully removed from the gel box. The DNA fragments or PCR fragments was visualized under UV trans-illuminator [16,17].

2.2.4 PCR mix components

The PCR mix components was made up of 12.5 µl of Taq 2 x Master Mix from New England Biolabs (M0270); 1 µl each of 10µm forward and reverse primer (Invitrogen, U.S.A™) (Table 1) [18,19]; 2 µl of DNA template and then made up with 8.5 µL Nuclease free water. The PCR reactions was executed as previously described [16,17].

3. RESULTS

3.1 Occurrence of Carbapenemase encoding Gene among *Pseudomonas aeruginosa* from different clinical sample

Genomic DNA extraction of twelve (12) clinical isolate of *Pseudomonas aeruginosa* are shown in Fig. 1.

Table 1. Primer sequence for Screening of carbapenemase encoding gene

Target gene	Primer sequence (5'-3')	Size (bp)	Cycling Condition
bla$_{IMP}$	F: GGAATAGAGTGGCTTAAYTCT R: CGGTTTAAYAAAACAACCACC	232	Initial denaturation at 94^{o}C x 5 mins, followed by 34 cycles of denaturation at 94^{o}C x 30 secs, 42 ^{o}C x 30 secs (annealing), 72 ^{o}C x 5 mins (elongation)
bla$_{VIM}$	F: GATGGTGTTTGGTCGCATA R: CGAATGCGCAGCACCAG	250	Initial denaturation at 94^{o}C x 5 mins, followed by 34 cycles of denaturation at 94^{o}C x 30 secs, 47 ^{o}C x 30 secs (annealing), 72 ^{o}C x 5 mins (elongation)
bla$_{KPC}$	F: CGTCTAGTTCTGCTTAGGCG R: CTTGTCATCCTTGTTAAACG	500	Initial denaturation at 94^{o}C x 5 mins, followed by 34 cycles of denaturation at 94^{o}C x 30 secs, 58.8 ^{o}C x 30 secs (annealing), 72 ^{o}C x 5 mins (elongation)
bla$_{OXA}$	F: GCGTGGTTAAGGATGAACAC R: CATCAAGTTCAACCCAACCG	550	Initial denaturation at 94^{o}C x 5 mins, followed by 34 cycles of denaturation at 94^{o}C x 30 secs, 52 ^{o}C x 30 secs (annealing), 72 ^{o}C x 5 mins (elongation)
bla$_{NDM}$	F: GGTTTGGCGATCTGGTTTTC R: CGGAATGGCTCATCACGATC	800	33 cycles at 95^{o}C x 3 mins, 65^{o}C x 30 secs followed by 1cycle at 72 ^{o}C x 10 mins

Among different sample source, *bla*$_{KPC}$ was detected 1(8.3 %) from Urine, wound swab 3(25.0 %) and Catheter tip 1(8.3 %) followed by *bla*$_{VIM}$ found 2(16.7 %) from Urine, wound swab 2(16.7 %) and Catheter tip 0(0.0 %) while *bla*$_{NDM}$ and *bla*$_{IPM}$ accounted 12 (100%) across all sample source as shown in Table 2.

3.2 Co-expression of Carbapenemase Encoding Gene among *Pseudomonas aeruginosa* from different clinical sample

Co-expression of *bla*$_{NDM}$ + *bla*$_{IMP}$ accounted 5(41.6 %), 5(41.6 %) and 2(16.7 %) in Urine, wound swab and Catheter tip respectively. Co-expression of *bla*$_{KPC}$ + *bla*$_{NDM}$ + *bla*$_{VIM}$ + *bla*$_{IMP}$ + *bla*$_{OXA}$ was only detected in urine 1(8.3 %) as shown in Table 3.

4. DISCUSSION

Both *bla*$_{IMP}$ and *bla*$_{NDM}$100% was the most predominant gene identified among *P. aeruginosa* while *bla*$_{VIM}$ 4(33.3 %) was the least identified gene. In contrast, several studies reported *bla*$_{VIM}$ gene as the most common MBL found in CR- *P. aeruginosa* [20,21] while in addition, the most prevalent carbapenemase encoding genes in *P. aeruginosa* are the VIM and IMP types; particularly, VIM-2 has become the dominant carbapenemase encoding gene type worldwide [22] but in accordance to a systemic review of carbapenase gene in *P. aeruginosa*, IMP followed by NDM have become the two most prevalent class B carbapenemases in worldwide *P. aeruginosa* isolates [11]. Also, in a previous study conducted in Iran bla$_{IMP}$ was reported as the most prevalent carbapenemase gene in *P. aeruginosa* isolates [23]. A retrospective screening of *P. aeruginosa* identified the *bla*$_{IMP}$ gene in 1992 in Japan [24]. Subsequent outbreaks due to the transferable drug resistance conferred by the gene were reported [25].

Although, *bla*$_{OXA}$ 41.7 % and *bla*$_{VIM}$ 33.3 % were detected. Their presence could be linked to the observed phenotypic MDR due to efflux pump and AmpC beta-lactamase overproduction. Similarly, a study conducted by Kao *et al.* [26] who analyzed the resistance mechanisms in 87 BSI-causing imipenem-resistant *P. aeruginosa* isolates collected in southern Taiwan between 2000 and 2010, revealed that carbapenemases (mainly VIM and OXA), active efflux pumps, and AmpC beta-lactamase overproduction were found in 10.3%, 74.4%, and 51.3% of the *P. aeruginosa* isolates, respectively [26]. Class D enzymes have a wide range of substrates and include OXA enzymes, which in general are not inhibited by traditional beta-lactamase inhibitors e.g., tazobactam, ticarcillin (used in this study) and may not be inhibited by new agents (e.g., vaborbactam, relebactam). OXA gene variants such as OXA-10 has been shown to exhibits ESBL activity and confers cefoxitin, ceftazidime and aztreonam resistance, while OXA- 31 confers resistance to cefepime [27,28] which are commonly use antibiotics in healthcare for the treatment enterobacteria infection in Nigeria.

P. aeruginosa isolates producing KPC are mainly identified in Europe and Asia [11] but detection of 5(41.7 %) KPC-producer in this study reveals a continental spread and may have been acquired from other enterobacteria such as *Klebsiella pnuemonaie* and *E. coli* were they are predominantly found.

The emergence of *bla*$_{NDM}$ and *bla*$_{IMP}$ encoding isolates can severely compromise the safety of vulnerable patients admitted to the hospital. As noted in this study, IMP and NDM producing *P. aeruginosa* are the carbapenemase-producing strains mainly circulating at our hospital environment. The widespread *P. aeruginosa* clone is often associated with poor clinical outcomes due to its multidrug resistance and virulence factors, representatively the cytotoxin *ExoU* causing necrotic cell death [11,29,30,31]. It has also been reported that New Delhi metallo-beta-lactamase (NDM producing strains are responsible for outbreaks throughout Europe, Asia, and South America [32] and its role in Pseudomonal infection in this hospital may not be underestimated.

Co-expression of *bla*$_{KPC}$ + *bla*$_{NDM}$ + *bla*$_{VIM}$ + *bla*$_{IMP}$ + *bla*$_{OXA}$ was only detected in one isolate 1(8.3 %). The carbapenem resistance determinants carried by *P. aeruginosa* are often encoded on plasmids, such as IncP type; class I integrons, such as those carrying the *bla*$_{OXA}$, *bla*$_{IPM}$, *bla*$_{KPC}$, *bla*$_{VIM}$ gene; and other mobile genetics elements, such as those associated with insertion sequences with a common region (ISCRs), which enhance the organism's ability to disseminate resistance among multiple species [7,11]. In addition, these isolates frequently carry additional resistance determinants that diminish the clinical utility of the beta-lactam, fluoroquinolones, aminoglycosides etc., as observed *in vitro* in this study. Carbapenemase producing *P. aeruginosa* are often resistant to all of these therapeutic options, thus making treatment failure a likely outcome.

Table 2. Occurrence of Carbapenemase encoding Gene among *Pseudomonas aeruginosa* from different clinical sample

Sample source	Urine(n=5)	Wound swab (n=5)	Catheter tip(n=2)	Total (n=12)
Isolate coding	**P1, P2, P3, P4, P5**	**P6, P7, P8, P9, P10**	**P11, P12**	
Gene				
bla$_{KPC}$	1(8.3 %)	3(25.0 %)	1(8.3 %)	**5(41.7 %)**
bla$_{NDM}$	4(33.3 %)	4(33.3 %)	4(33.3 %)	**12(100 %)**
bla$_{VIM}$	2(16.7 %)	2(16.7 %)	0(0.0 %)	**4(33.3%)**
bla$_{IMP}$	4(33.3 %)	4(33.3 %)	4(33.3)	**12(100%)**
bla$_{OXA}$	3(25.0 %)	1(8.3 %)	1(8.3 %)	**5(41.7 %)**

KEY: n - number of isolate, bla$_{KPC}$*-Klebsiella pneumoniae carbapenemase, bla*$_{IMP}$*-Imipenemases, bla*$_{VIM}$*-Verona integron Metallo-beta-lactamase, bla*$_{NDM}$*-New Delhi Metallo-beta-lactamase, bla*$_{OXA}$*-Oxacillinase.*

Table 3. Co-expression of Carbapenemase Encoding Gene among *Pseudomonas aeruginosa* from different clinical sample

Sample source	Urine (n=5)	Wound swab (n=5)	Catheter tip (n=2)	Total (n=12)
Isolate coding	**P1, P2, P3, P4, P5**	**P6, P7, P8, P9, P10**	**P11, P12**	
Co-expression				
bla$_{NDM}$ + *bla*$_{IMP}$	5(41.6 %)	5(41.6 %)	2(16.7 %)	**12(100 %)**
bla$_{KPC}$+*bla*$_{NDM}$+ *bla*$_{VIM}$+ *bla*$_{IMP}$+*bla*$_{OXA}$	1(8.3 %)	0(0.0 %)	0(0.0 %)	**1(8.3%)**

KEY: %-Percentage, n-Number of isolate, bla$_{KPC}$*-Klebsiella pneumoniae carbapenemase, bla*$_{IMP}$*-Imipenemases, bla*$_{VIM}$*-Verona integron Metallo-beta-lactamase, bla*$_{NDM}$*-New Delhi Metallo-beta-lactamase, bla*$_{OXA}$*-Oxacillinase*

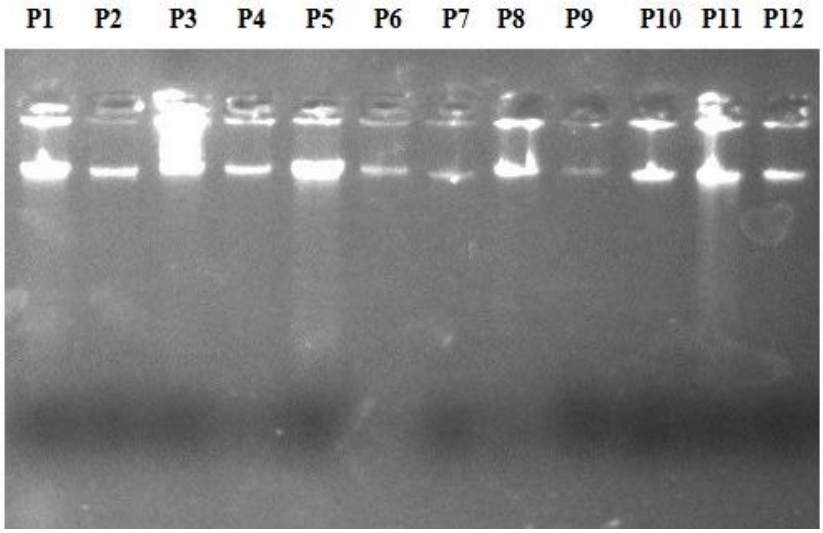

Fig. 1. Genomic DNA extraction of *Pseudomonas aeruginosa*

5. CONCLUSION

The current study provides baseline information on the occurrence of carbapenemase-encoding genes carried by *P. aeruginosa*. The expression of these genes could be the mainstay of phenotypic MDR. Therefore, physicians, other medical professionals, researchers, and public health policymakers must be informed about the spread of this carbapenemase encoding genes, as it may help in appropriate initiation of antimicrobial therapy that reduces the morbidity and mortality of infection among the patients particularly admitted in critical care units. Also, in admission healthcare facilities where carbapenemase encoding genes are spreading, strict infection prevention and control strategies, as well as antimicrobial stewardship programs, are highly desirable.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICAL APPROVAL

The approval for this study was conveyed with the Ethical clearance number SMOH/ERC/043/21 obtained from the Ministry of Health Ebonyi State's research and ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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