1 ***Original Research Article***

2 *oprD* genes detected in *Pseudomonas* 3 *aeruginosa* isolates from a teaching hospital 4 but lost in a carbapenem-resistant strain

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7 **ABSTRACT**

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Aims: The aims of the study were to evaluate the multidrug resistance profile and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* clinical isolates using phenotypic and genotypic methods.

Study Design: A descriptive laboratory based study.

Place and Duration of Study: Microbiology Laboratory, Ondo State University of Science and Technology, Okitipupa, and Biotechnology Laboratory, Ladoke Akintola University of Technology, Osogbo, Nigeria, between June 2017 and November 2018.

Methodology: Ten *P. aeruginosa* isolates were recovered from patients at Lagos University Teaching Hospital, and susceptibilities to imipenem (10µg), meropenem (10µg) and a panel of antibiotics were performed by the disk diffusion method. Genotypic methods including Polymerase Chain Reactions (PCR) and agarose gel electrophoresis were carried out according to established protocols. *oprD* and ^{bla}*IMP* gene primers were used for the PCR amplicication.

Results: Fifty percent (50%) of the isolates showed multiple drug resistance. Four isolates (40%) were carbapenem resistant (CR). *oprD* gene was detected in 90% (9/10) of the isolates. 75% (3/4) of CR strains were among the strains showing *oprD* gene. 25% (1/4) CR strain (PA1421) was *oprD* negative. Loss or mutation of *oprD* gene seems to be the mechanism of carbapenem resistance in strain PA1421.

Conclusion: Loss or mutation of *oprD* gene was identified in this study as a mechanism of carbapenem resistance. *oprD* gene encodes the outer membrane protein (OprD) porin in *P. aeruginosa* whose deficiency confers resistence to carbapenems, especially imipenem. Surveillance of the antimicrobial susceptibility patterns of *P. aeruginosa* is of critical importance in understanding new and emerging resistance trends, reviewing antibiotic policies and informing therapeutic options.

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10 *Keywords:* *oprD*, ^{bla}*IMP*, carbapenem resistance, Nigeria, *Pseudomonas aeruginosa*.

1. INTRODUCTION

The World Health Organization (WHO) in 2017 released a global priority pathogens list (global PPL) of antibiotic-resistant bacteria to help in prioritizing the research and development of new and effective antibiotic treatments. The list contains three categories of antibiotic-resistant pathogenic bacteria categorized as Priority 1 (Critical), Priority 2 (High), and Priority 3 (Medium). Carbapenem-resistant *Pseudomonas aeruginosa* rank second in the critical list which includes multidrug resistant bacteria that pose a particular threat in hospitals, nursing homes, and among patients whose care requires devices such as ventilators and blood catheters, causing severe and often life threatening infections such as bloodstream infections and pneumonia [1].

Carbapenems, such as imipenem and meropenem are often used as last resort antibiotics for the treatment of multidrug resistant *Pseudomonas aeruginosa* infections [2]. Of all the beta-lactams, carbapenems possess the broadest spectrum of activity and the greatest potency against bacteria, and so are often reserved for more severe infections or used as 'last-line' agents.

Like all beta-lactams, carbapenems inhibit bacterial cell wall synthesis by binding to the penicillin-binding proteins and interfering with cell wall formation. Carbapenems have excellent activity against a broad spectrum of aerobic and anaerobic bacteria, and are notable for their ability to inhibit beta-lactamase enzymes. They are usually employed in serious infections such as intra-abdominal, skin and soft tissue that are resistant to first line antibiotics [3].

Pseudomonas aeruginosa is an opportunistic pathogen associated with a range of healthcare associated infections that can be particularly severe in immune-compromised patients, and is extraordinary because it has the potential to overcome the activity of almost all the available antibiotics [4], and the ability to acquire genes encoding resistance determinants. The development of carbapenem resistance among *P. aeruginosa* strains has been attributed to multiple factors such as plasmid or integron-mediated carbapenemases, increased expression of efflux systems, reduced porin expression and increased chromosomal cephalosporinase activity [3]. The main reported mechanism of resistance to carbapenems involves the loss or downregulation of OprD porin from the outer membrane through deletions, mutations or insertions in the *oprD* gene [5].

The increasing isolation in healthcare settings of *P. aeruginosa* strains resistant to carbapenems has raised a global alarm which necessitates constant surveillance and more detailed research. In the present study, the authors used phenotypic tests and molecular techniques to identify the resistance determinants in carbapenem-resistant *P. aeruginosa* isolated from hospital patients.

2. MATERIAL AND METHODS

2.1. Sampling

Ten (10) clinical isolates of *Pseudomonas aeruginosa* identified with Microbact 24E (Oxoid Ltd, Cambridge, UK.) were obtained from the Microbiology Laboratory of Lagos University Teaching Hospital (LUTH) in June, 2018. The isolates were code-named as PA40, PA1340, PA1349, PA1357, PA1380, PA1421, PA1423, PA1425, PA1656, and PA1792.

2.2. Antimicrobial susceptibility testing

Susceptibilities of the isolates to imipenem (10 µg), meropenem (10 µg), colistin sulphate (10 µg), ofloxacin (5 µg), gentamicin (10 µg), and ceftazidime (30 µg) (Oxoid Ltd, Cambridge, UK.) were determined according to Clinical and Laboratory Standard Institute guidelines [6]. A pure culture of each *P. aeruginosa* isolate was used. Four to five colonies of each isolate were transferred to 5 mL of nutrient broth and were cultured overnight at 35°C. The overnight cultures were then diluted with sterile saline (0.85% NaCl) in Bijou bottles, and their turbidity was adjusted to 0.5 McFarland standards. The inocular were spread with a sterile cotton wool swab on Mueller–Hinton agar. The antibiotic sensitivity disks were applied with sterile forceps, and the agar plates were incubated for a full 24 h at 35°C aerobically. The inhibition zone diameter (ZD) for each isolate was measured and interpreted as “Resistant”, “Intermediate” or “Sensitive” using a standardized table according to CLSI breakpoints [6].

2.3. DNA extraction

Deoxyribonucleic acid (DNA) extraction from each *P. aeruginosa* isolate was carried out by modification of the simple crude extraction methods previously described for *Salmonella enterica* [7] and *Streptococcus pneumoniae* [8]. Twenty-four-hour-old pure colonies of each *P. aeruginosa* isolate were suspended in 500 µL of Tris-buffer (1x) in appropriately labelled Eppendorf tubes (Eppendorf North America, Hauppauge, NY, USA). The cells were washed three times in sterile distilled water while vortexing and centrifuging at 10, 000 rpm. Tubes were covered and sealed with paraffin tape to prevent accidental opening. After the last washing, the suspensions were boiled for 10 min in a water-bath at 100^{o}C and then cold shocked in ice for 2 min. Thereafter, they were centrifuged at 14000 rpm for 5 min to obtain the supernatant. The supernatants containing the DNA were stored at 4°C before use. Aliquots of 2 µL of template DNA were used for PCR.

2.4. Polymerase Chain Reactions (PCR)

2.4.1. Primers and Deoxynucleases (dNTPs)

Outer membrane protein D gene primer (oprD F and oprD R); and imipenemase gene primer (^{bla}IMP-1F and ^{bla}IMP-1R) were obtained from Inqaba Biotec West Africa. Deoxynucleases (dNTPs) solution was obtained from BioLabs (New England).

2.4.2. Preparation of Mastermix for amplification of *oprD* gene

The Mastermix for amplification of *oprD* gene was constituted by using a microliter pipette to add the required reagents into an Eppendorf tube (Table 1). The reagents were mixed to obtain a uniform mixture using a vortex mixer and centrifuge. The same procedure was used to prepare a separate Mastermix for the amplification of ^{bla}*IMP* gene.

Table 1: Constituents of Mastermix for PCR Amplification of *oprD* gene

Constituent	Volume (µL)
Nuclease-free water	110 µL
PCR buffer	22 µL
$MgCl_2$ solution	11 µL
DNTP solution	8.8 µL
oprDF (forward primer)	5.5 µL
oprDR (reverse primer)	5.5 µL
Taq polymerase	2.2 µL

2.4.3. Protocols for PCR

Eighteen microliters (18µL) of the Mastermix was introduced into each of the PCR tubes and 2µL of DNA was added. The PCR tubes were loaded into a thermal cycler (Prime) and subjected to the following conditions for the different primers as previously described [9-12].

The *oprD* gene was amplified with the following primers:

oprDF 5'-ATGAAAGTGATGAAGTGGAG-3'
oprDR 5'-CAGGATCGACAGCGGATAGT-3'

Product= 1329bp (Accession nos. KT736319/KT728193/MH135304)

PCR conditions were: 1 cycle of initial denaturation at 94^{o}C for 2min; 30 cycles of (denaturation at 94^{o}C for 45 sec, annealing at 55^{o}C for 45 sec, elongation at 72^{o}C for 45 sec); and final elongation for 72^{o}C for 5min. Expected size of amplicon 1329-bp.

^{bla}*IMP* gene was amplified with the following primers:

^{bla}IMP -1F 5'-TGA GCA AGT TAT CTG TAT TC-3'
^{bla}IMP -1R 5'-TTA GTT GCT TGG TTT TGA TG-3'

PCR conditions were: 1 cycle of initial denaturation at 94°C for 2mins; 30 cycles of (denaturation at 94°C for 1min, annealing at 56°C for 1min, elongation at 72°C for 2 min); and final elongation at 72 °C for 10 min. Expected size of amplicon 749-bp.

2.5. Agarose Gel Electrophoresis

At the completion of the amplification, PCR products were resolved on I% agarose gel prepared by dissolving 1g of agarose powder in 100 ml of 1x Tris-borate-EDTA (TBE) buffer solution inside a clean conical flask. The 1% agarose solution was heated in a microwave oven for 2-3 minutes and was observed for clarity which was an indication of complete dissolution. The mixture was then allowed to cool to about 50 °C after which 0.5 µl of 1 µg/mL ethidium bromide (Sigma-Aldrich, St Louis, MO, USA) was added. It was allowed to cool further and then poured into a tray sealed at both ends with support to form a mould with special combs placed in it to create wells. The comb was carefully removed after the gel had set and the plate was placed inside the electrophoresis tank which contained 1x TBE solution. A 5 µl of amplicon was mixed with 5 µl of Orange G (loading buffer) and loaded to the well of the agarose gel. The power supply was adjusted to 100 volts for 25 minutes. For each run, a 100 base-pair molecule weight DNA standard (size marker) was used to determine the size of each PCR product. The DNA bands were then visualized with a short wave ultraviolet trans-illuminator and photographed using gene gel bio-imaging system (SynGene Bioimaging System; Syngene UK, Cambridge, UK). The PCR product was then analyzed.

2.6. Data analysis

Data obtained in the study was analyzed using the following equations where 'A' is antibiotic tested; 'CS' means carbapenem-susceptible; 'CR' means carbapenem-resistant:

$$\textit{Percentage resistance to antibiotic A} = \frac{\textit{number of isolates resistant to A}}{\textit{total number of isolates}} \times 100 \quad (1)$$

$$\textit{Percentage CS isolates having OprD gene} = \frac{\textit{number of CS isolates}}{\textit{total number of isolates}} \times 100 \quad (2)$$

$$\textit{Percentage CR isolates having OprD gene} = \frac{\textit{number of CR isolates}}{\textit{total number of isolates}} \times 100 \quad (3)$$

3. RESULTS AND DISCUSSION

3.1. Results of antimicrobial susceptibility screening

The results of antimicrobial susceptibility testing of *Pseudomonas aeruginosa* isolates are presented in Table 2. The diameters of zones of inhibition (IZD) in mm were interpreted using updated CLSI (2017) breakpoints [6]. IZD for colistin sulphate was interpreted according to CLSI (2014) breakpoints giving ≥11 as 'sensitive' and ≤10 'resistant' [13]. Multidrug resistance (MDR) was taken as resistance to at least three classes of antibiotics. In this study, four isolates (PA1340, PA1380, PA1421 and PA1656) representing 40% of all the isolates, were resistant to imipenem and meropenem. The

isolates showed resistances to ofloxacin (50%), gentamicin (100%), and ceftazidime (100%). All the isolates were susceptible to colistin sulphate.

Table 2: Zones of Inhibition (mm) produced by antibiotics against *Pseudomonas aeruginosa*

Isolate	IMP (10µg)	MEM (10µg)	CT (10µg)	OFL (5µg)	GEN (10µg)	CAZ (30µg)	MDR
PA40	46	40	19	32	10	0	-
PA1340	0	0	16	0	0	0	√
PA1349	30	38	16	35	10	0	-
PA1357	30	41	17	26	10	0	-
PA1380	12	0	17	0	0	0	√
PA1421	0	0	13	0	0	0	√
PA1423	28	32	15	17	10	0	-
PA1425	35	44	19	28	9	0	-
PA1656	11	0	19	0	0	0	√
PA1792	34	40	20	0	0	0	√
S (%)	60	60	100	40	0	0	
I (%)	0	0	0	10	0	0	
R (%)	40	40	0	50	100	100	

Keys: IMP- imipenem, MEM- meropenem, CT- colistin sulphate, OFL- ofloxacin, GEN- gentamicin, CAZ- ceftazidime, % S- percentage sensitivity to antibiotic, % I- percentage intermediate to antibiotic, % R- percentage resistance to antibiotic. √ - multidrug resistant isolate.

3.2. Results of Polymerase Chain Reaction (PCR)

3.2.1 Detection of *OprD* genes in *P. aeruginosa* isolates

The results of agarose gel electrophoresis of PCR products of *P. aeruginosa* isolates are shown in Figure 1. Nine of the isolates were positive for *oprD* gene which showed bands corresponding to 1329 base pairs. Strain PA1421 (well 8) did not show any band corresponding to 1329 base pairs and consequently was interpreted as *oprD* negative. The ladder (L) is a 100 base-pair molecular weight DNA standard (size marker).

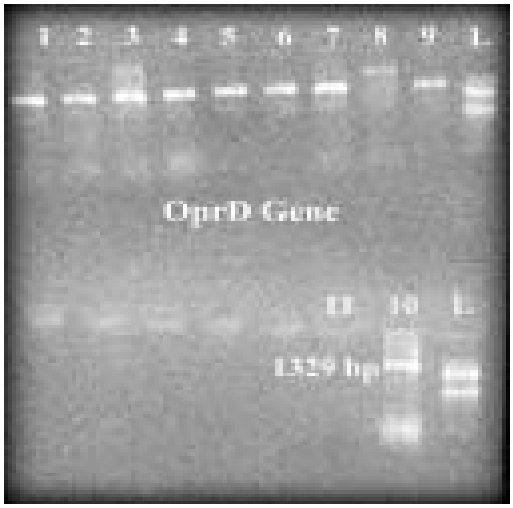

Figure 1: *oprD* gene (1329 bp) detected in nine *P. aeruginosa* isolates. Strain PA1421 (well 8) was *oprD*-negative.

3.2.2 Correlation of carbapenem susceptibility, multiple drug resistance (MDR), and *oprD* detection

The correlation of carbapenem resistance and multiple drug resistance (A); carbapenem resistance and *oprD* -positive (B); carbapenem resistance and *oprD* -negative (C); carbapenem susceptible and *oprD* -positive (D) are shown in Table 3.

(A) Carbapenem resistance correlated with multiple drug resistance in 40% of the isolates (PA1340, PA1380, PA1421, PA1656).

(B) Carbapenem resistance correlated with *oprD* –positive in 30% of the isolates (PA1340, PA1380, PA1656).

(C) Carbapenem resistance correlated with *oprD* –negative in 1.0 % of the isolates (PA1421).

(D) Carbapenem susceptible correlated with *oprD* –positive in 60% of the isolates (PA40, PA1349, PA1357, PA1423, PA1425, PA1792).

Table 3: Correlation of multiple drug resistance (MDR), carbapenem resistance and *oprD* detection

Isolate code	Imipenem	Meropenem	MDR	*oprD*	Correlation code
PA40	S	S	-	+	D
PA1340	R	R	+	+	A, B
PA1349	S	S	-	+	D
PA1357	S	S	-	+	D
PA1380	R	R	+	+	A, B
PA1421	R	R	+	-	A, C

PA1423	S	S	-	+	D
PA1425	S	S	-	+	D
PA1656	R	R	+	+	A, B
PA1792	S	S	+	+	D

Keys: S susceptible, R resistant, MDR multidrug resistant.

The findings of the present study are similar to reports of previous authors. In India, Shashikala *et al*. (2006) reported a 10.9% resistance to imipenem and meropenem [14]. Yin *et al*. (2018) in China reported higher rates of resistance such as 64.3% to imipenem and 67.9% to meropenem [15]. These findings corroborate global reports of increasing carbapenem resistance among *P. aeruginosa* clinical isolates.

Fifty percent (50%) of *P. aeruginosa* isolates were multidrug resistant (MDR). MDR is very common in *P. aeruginosa* isolates from hospitals and other sources and a major cause of concern in the health sector in Nigeria [16].

All the isolates were susceptible to colistin sulphate (100%), despite poor diffusion of colistin in agar medium. This seems to agree with the current use of colistin as the 'last line' antibiotic for multidrug-resistant Gram negative bacteria pathogens [17].

Pseudomonas aeruginosa isolates showed 60% sensitivity to carbapenems in the present study. A similar report from Iraq finds imipenem the best antibiotic against MDR *P. aeruginosa* from clinical sources (88.4% sensitivity) and from sewage (96.7% sensitivity) [18]. These findings are in consonance with several reports that carbapenems are very useful as last resort beta-lactams for multiple-drug resistant *P. aeruginosa* infections. However, emerging resistance to carbapenems limits therapeutic options. Therefore periodic surveillance of the resistance pattern is critical for the selection of an appropriate empiric antimicrobial agent [14].

In the PCR, *oprD* gene with a band size of 1329-bp was detected in nine of the isolates but was not detected in one strain PA1421 (Plate 1). Detection of *oprD* gene in three out of four (75%) of carbapenem resistant (CR) strains indicates the presence of outer membrane protein (OprD), an evidence that loss or mutation of *oprD* was not the mechanism of resistance in these strains (PA1340, PA1380, PA1656). One out of four (25%) of CR strains showed a loss or mutation of *oprD* known to result in carbapenem resistance and which seems to be the mechanism of carbapenem resistance in the strain (PA1421). *oprD* was detected in 100% (6/6) of carbapenem susceptible (CS) strains.

P. aeruginosa can use a combination of *chromosomally* encoded and /or plasmid encoded mechanisms to evade carbapenem therapy. Yin *et al*. (2018) found the main mechanism associated with carbapenem resistance was mutational inactivation of *oprD* in 88.65% of samples [15].

Carbapenems enter into the periplasmic space of *P. aeruginosa* through the OprD outer membrane porin. The porin loss probably by a mutational event of the *oprD* gene leads to imipenem resistance [19]. Furthermore, in strains with *oprD* downregulation, reduced susceptibility to meropenem is observed while other beta-lactams are not affected [20-21]. Diminished expression or loss of the *oprD* gene is rather frequent during imipenem treatment [22].

OprD is the outer membrane protein in *P. aeruginosa* whose deficiency confers resistence to carbapenems, especially imipenem. Functional studies have revealed that loops 2 and 3 in the OprD protein contain the entrance and/or binding sites for imipenem. Therefore any mutation in loop 2 and/or loop 3 that causes conformation changes could result in carbapenem resistance. OprD is also a common channel for some amino acids and peptides. Because of its hypermutability and highly regulated properties, OprD is thought to be the most prevalent mechanism for carbapenem resistance in *P. aeruginosa* [23]. In a study in Iran by Shariati *et al*. (2018), PCR assay using *oprD*-specific primers demonstrated that 10.52% (10/95) of imipenem-resistant *P. aeruginosa* isolates harbored an insertion sequence (IS) element in the *oprD* gene which inactivates the gene. Insertional inactivation of *oprD* gene resulted in a reduction of carbapenem susceptibility and loss of OprD production [10].

The ^{bla}*IMP* gene was not detected in any of the isolates in the present study. On a similar report, Al-Ouqaili *et al*. (2018) detected *oprD* in 44.4% of clinical isolates of *P. aeruginosa* but did not detect ^{bla}*IMP* in any of the isolates [24]. The failure to amplify or detect ^{bla}*IMP* gene could arise from a number of factors which include loss of the genes in the isolates, or wrong PCR or electrophoresis conditions. ^{bla}*IMP* genes encodes the metallo-beta-lactamase IMP.

4. CONCLUSION

The results of this study reveal the increasing carbapenem resistance of *P. aeruginosa* isolates in Nigeria, similar to reports from other countries globally [14-15; 18]. The high rate of sensitivity (100%) of the isolates to colistin sulphate is evidence that the drug is effective as a last resort drug against MDR *P. aeruginosa*. The findings of this study corroborate other reports that a loss or mutation of *OprD* is the main mechanism of carbapenem resistance, especially during imipenem treatment [20-21; 23].

Healthcare-associated infections caused by multi-drug resistant *P. aeruginosa* are a significant cause of morbidity and mortality in hospital settings. *P. aeruginosa* strains harboring carbapenem resistance mechanisms limit therapeutic options because carbapenem resistance is associated with resistance to other antibiotic classes. Therefore, surveillance of the antimicrobial susceptibility patterns of *P. aeruginosa* is of critical importance in understanding new and emerging resistance trends, reviewing antibiotic policies and informing therapeutic options. Increasing CR in *P. aeruginosa* isolates from hospital patients calls for greater commitment in research and drug development.

COMPETING INTERESTS

Authors declare that no competing interests exist.

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