oprD Genes Detected in Pseudomonas aeruginosa Isolates from a Teaching Hospital but Lost in a Carbapenem-Resistant Strain

Eucharia E. Nmema1*, Chioma S. Osuagwu2 and Eunice N. Anaele3

1Department of Biological Sciences, Ondo State University of Science and Technology, P.M.B. 353, Okitipupa, 350002, Ondo State, Nigeria.
2Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos, P.M.B. 12003, Idi-Araba, Lagos 100001, Nigeria.
3South-East Biotechnology Center, University of Nigeria, Nsukka 410001, Enugu State, Nigeria.

Authors’ contributions

This work was carried out in collaboration among all authors. Authors EEN, CSO and ENA designed the study. Author EEN wrote and performed the protocol, statistical analysis, and wrote the first draft of the manuscript. Authors EEN, CSO and ENA managed the literature searches and analyses of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMMR/2019/v29i930122

Received 11 February 2019
Accepted 22 April 2019
Published 08 May 2019

ABSTRACT

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 (10 µg), meropenem (10 µg) 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 βlactamase genes were used for the PCR amplification.

**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 resistance to carbapenem, 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.

**Keywords:** OprD, βlactamase; 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, carbapenem 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 immunocompromised 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. MATERIALS 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
isolation 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 inocula 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 pneumonia [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, Hauppaug, 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°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 (blaIMP-1F and blaIMP-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 blaIMP gene.

Table 1. Constituents of mastermix for PCR amplification of oprD gene

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>110 µL</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>22 µL</td>
</tr>
<tr>
<td>MgCl2 solution</td>
<td>11 µL</td>
</tr>
<tr>
<td>DNTP solution</td>
<td>8.8 µL</td>
</tr>
<tr>
<td>OprDF (forward primer)</td>
<td>5.5 µL</td>
</tr>
<tr>
<td>OprDr (reverse primer)</td>
<td>5.5 µL</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>2.2 µL</td>
</tr>
</tbody>
</table>

2.4.3 Protocols for PCR

Eighteen microliters (18 μL) of the Mastermix was introduced into each of the PCR tubes and 2 μL 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’-ATGAAAGTGATGAGTGAGG-3’
- oprDR 5’-CAGGATCGACAGCGGATAGT-3’

Product= 1329bp (Accession nos. KT736319/HT728193/MH135304)  

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

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

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:

Percentage resistance to antibiotic A = (number of isolates resistant to A / total number of isolates) × 100

Percentage CS isolates having OprD gene = (number of CS isolates / total number of isolates) × 100

Percentage CR isolates having OprD gene = (number of CR isolates / total number of isolates) × 100

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...

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

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
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.

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 Fig. 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).

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.

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

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Imipenem</th>
<th>Meropenem</th>
<th>MDR</th>
<th>oprD</th>
<th>Correlation code</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA40</td>
<td>S</td>
<td>S</td>
<td>-</td>
<td>+</td>
<td>D</td>
</tr>
<tr>
<td>PA1340</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>+</td>
<td>A, B</td>
</tr>
<tr>
<td>PA1349</td>
<td>S</td>
<td>S</td>
<td>-</td>
<td>+</td>
<td>D</td>
</tr>
<tr>
<td>PA1357</td>
<td>S</td>
<td>S</td>
<td>-</td>
<td>+</td>
<td>D</td>
</tr>
<tr>
<td>PA1380</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>+</td>
<td>A, B</td>
</tr>
<tr>
<td>PA1421</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>-</td>
<td>A, C</td>
</tr>
<tr>
<td>PA1423</td>
<td>S</td>
<td>S</td>
<td>-</td>
<td>+</td>
<td>D</td>
</tr>
<tr>
<td>PA1425</td>
<td>S</td>
<td>S</td>
<td>-</td>
<td>+</td>
<td>D</td>
</tr>
<tr>
<td>PA1656</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>+</td>
<td>A, B</td>
</tr>
<tr>
<td>PA1792</td>
<td>S</td>
<td>S</td>
<td>+</td>
<td>+</td>
<td>D</td>
</tr>
</tbody>
</table>

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

The findings of the present study are similar to reports of previous authors. In India, Shashikala et al. [14] reported a 10.9% resistance to imipenem and meropenem. Yin et al. [15] in China reported higher rates of resistance such as 64.3% to imipenem and 67.9% to meropenem. 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. [15] found the main mechanism associated with carbapenem resistance was mutational inactivation of oprD in 88.65% of samples.

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 resistance 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. [10], 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.

The blaIMP gene was not detected in any of the isolates in the present study. On a similar report, Al-Ouqaili et al. [24] detected oprD in 44.4% of clinical isolates of P. aeruginosa but did not detect blaIMP in any of the isolates. The failure to amplify or detect blaIMP gene could arise from a number of factors which include loss of the
genes in the isolates, or wrong PCR or electrophoresis conditions. blaIMP 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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


© 2019 Nmema et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.