GENOMIC INVESTIGATION OF *bla-PAO* AND *bla-OXA50*IN MULTIDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA* FROM CLINICAL SAMPLES IN ABEOKUTA, OGUN STATE,Nigeria

ABSTRACT

Pseudomonas aeruginosa is one of the pathogens of human concern with high intrinsic multi-drug resistance capabilities. The genomic investigation of blaPAO and blaOXA-50 was done on multi drug-resistant *Pseudomonas aeruginosa* that were also resistant to carbapenem among the isolates collected from a total of 128 clinical samples in Abeokuta, Nigeria. *Pseudomonas aeruginosa* isolates were obtained from pure culture, and profiled for antibiogram by disc diffusion method. Genomic DNA from isolates were typed for blaPAO and blaOXA-50 with PCR. A total of 75 samples (58.6%) yielded the growth of bacterial isolates. Bacteria isolated were *Escherichiacoli* (18.8%),*Klebsiella pneumoniae* (16.4%), *Staphylococcus aureus* (8.6%,), *Pseudomonas aeruginosa* (7.8%), *Streptococcus pnuemoniae*(3.1%), *Proteus mirabilis* (2.3%), and *Enterobacter aerogenes* (1.6%). Only ten (30%) isolates were confirmed to be *Pseudomonas aeruginosa*. All the *P. aeruginosa* isolates were resistant to ampicillin, cloxacillin, erythromycin, and tetracycline. Out of these ten multidrug *Pseudomonas aeruginosa* isolates, only three (30%) were resistant to carbapenems. Only two of these isolates expressed blaOXA-50 and blaPAO, while one possessed only blaPAO. Close Continuous monitoring of these antibiotic-resistant pathogens and hospital surveillance needs to be adopted to reduce their spread to other healthcare facilities.

Keywords:*Pseudomonas aeruginosa;* antibiotic resistance; blaPAO; blaOXA-50

INTRODUCTION

Pseudomonas aeruginosa is one of the most common pathogens isolated from patients who have been hospitalized longer than one week and it is a leading cause of nosocomial infections in hospital settings and death cases have also been reported (1). *P. aeruginosa* are ubiquitous and are found in several environmental niche and can spread to patients and healthcare providers in clinical settings particularly when exposed to contaminated clinical instruments, surfaces, beddings, water or soil. There is high risk of infection with patients on ventilators or other medical devices such as intravenous catheters (2). Nosocomial *P.aeruginosa* infection include bloodstream infection, pneumonia, urinary tract infections and surgical wound infection. These infections mostly affect hospitalized patients especially those with weak immune systems and those on long term treatments (3). *P. aeruginosa* has been recognized to have survival and adaptation abilities in a wide range of environments such as soil, water, sewage and hospitals (4). Despite therapy the mortality due to nosocomial is approximately 70%, *P. aeruginosa* develops resistance to most of antibiotics thereby preventing the selection of appropriate treatment (5). "Multi Drug Resistant *P. aeruginosa* (MDRPA) is a condition that bacteria resistant to three or more classes of antibiotics such as penicillins, cephalosporins, monobactam, carbapenem, aminoglycosides and fluoroquinolones. Inappropriate antibiotics administration can cause *P. aeruginosa* resistant to several classes of antibiotics" (6, 7). "*P. aeruginosa* nosocomial infections is generally difficult to treat because of the possibility intrinsic resistance and its ability to obtain faster resistance mechanism against many groups of antimicrobials" (8).

"*Pseudomonas aeruginosa* are both invasive and toxigenic bacteria and has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance" (9). "This organism has been incriminated in cases of meningitis, septicaemia, pneumonia, ocular and burn

infections”(10). “Wound infections related to burn patients often leads to bacteraemia. Different conditions such as severe neutropenia, mucosal ulcers, and malignancies lead to a risk for bacteraemia” (11, 12). “*Pseudomonas aeruginosa* is most commonly found in cystic fibrosis patients. The abnormal airway epithelia of these patients allow long-term colonization by this bacterium and, once they get infected, they rarely fade away and lead to chronic lung diseases” (13).**“***Psuedomonas. aeruginosa* possess various *virulence* genes that contribute to its pathogenicity such as exotoxin The blaOXA -50 gene (formerly known as the PA5514 gene) is an oxacillinase gene identified in Sicily in the genome of Pseudomonas PAO1 isolate” (14). “It has been reported that blaOXA – 50 naturally exists in all *P. aeruginosa* and does not appear to have been acquired based on the similar GC% content of the blaOXA – 50 gene to the overall *P. aeruginosa* genome” (15).“Carbapenems are β-lactam antibiotics that consist of a four-membered β-lactam ring fused with a secondary five-membered thiazolidine ring through the nitrogen and adjacent tetrahedral carbon atom. Unlike other β-lactams, carbapenems have two substitutions, at position one there is a substitution of sulfur for a carbon atom and at the fourth position of the thiazolidinic moiety, a carbon is substituted for a sulfone” (16-18). The aim of our current study is to investigate the prevalence of muti-drug resistance *P. aueregino*sa possessing the beta lactamase bla-OXA-50 and blaPAO1 genes isolated from clinical samples in Abeokuta, Ogun State.

MATERIALS AND METHODS

Sampling, Isolation and Identification

One hundred and twenty-eight clinical samples from Federal Medical Centre, Abeokuta, in southwest Nigeria were analyzed between June 2023 and January 2024. *P. aeruginosa* isolates obtained from the clinical sampleswere preserved in semi-solid Brain Heart Infusion (BHI) (Oxoid, Basingstoke, UK) supplemented with glycerol and re-characterized for confirmation following standard biochemical methods previously described (19).

Antimicrobial Susceptibility

The susceptibility profile of antibiotics commonly prescribed for *Pseudomonas aeruginosa* infections was determined using Kirby-Bauer disc diffusion in accordance with CLSI

recommendations and guidelines (20, 21). Overnight culture from cetrimide agar was sub-cultured on BHI and further incubated for 24 h at 37 ◦C. Bacterial suspension of 0.5 MacFarland turbidity was spread on BHI using sterile swab stick. Twelve different antibiotics with different disc concentrations, such as Gentamycin (Gen) 10 µg/disc, Erythromycin (Ery) 15 µg/disc, Ceftriaxone (Cef) 30 µg/disc, Imipenem (Imp) 10 µg/disc, Meropenem (Mem) 10 µg/disc, Tetracycline (Tet) 30 µg/disc, Cefuroxime (Str) 30 µg/disc, Cloxacillin 30 µg/disc (Cxc), Ampicillin (Amp), 30 µg/disc, Cefuroxime (Cxm) 30 µg/disc, Ceftazidime (Caz) 30 µg/disc, Cefepime (Cef) 30 µg/disc and Ciprofloxacin (Cip) 5 µg/disc were used in this study. The antimicrobial sensitivity test of each isolate was carried out as described by the Kirby-Bauer disc diffusion method. The turbidity of the bacterial suspensions was compared with 0.5 Macfarland's barium sulfate standard solution. The standardized bacterial suspension was inoculated on Muller Hinton Agar (Lab M Laboratories, Mumbai, India) and left to dry for 10 minutes before placing the antimicrobial sensitivity discs. Antibiotic-impregnated discs of 8 mm in diameter were used for the test. After incubation, the diameter of the zone of inhibition was measured and compared with the zone diameter interpretative chart (20, 21) to determine the sensitivity of the isolates to antibiotics. The standard strain, *P. aeruginosa* ATCC 27853, was used as a control. Isolates showing resistance to at least one agent in more than three classes of the antibiotic group were classified as multi-drug resistant *P. aeruginosa* (MDR P. aeruginosa) according to Magiorakos*et al.* (22). Multi antibiotic resistance index (MARI), was calculated for each isolated tested, using the formula below

MARI = No of resistant antibiotics ÷ Total number of antibiotics tested

Chromosomal DNA Extraction

Purified genomic bacterial DNA was extracted from overnight cultures of the three multidrug-resistant Pseudomonas aeruginosa isolates after growth on Tryptic Soy agar (TSA) medium using a genomic DNA mini kit (QIAGEN, QIAamp®, USA) according to the manufacturer's instructions. This serves as the template DNA. The concentration of the eluted DNA was measured using a NanoDrop 2000 spectrophotometer.

Polymerase Chain Reaction

The pure DNA of each of the three different *Pseudomonas aeruginosa* was subjected to the polymerase chain reaction (PCR), with the genes blaPAO and blaOXA-50 being targeted. The PCR reaction was performed with primer sets blaPAO forward F-TGCCTGGTAGTGGGGGATAA, reverse F-TGCCTGGTAGTGGGGGATAA, and blaOXA50 forward F-AATCCGGCGCTCATCCATC reverse R: GGTCGGCGACTGAGGCGG a total volume of 50 µL, containing 25 ng of DNA template, 10 mMTris-HCl, 50 nmolKCl, 1.5 mM MgCl2, 200 µM dNTP (Fermentas), 12.5 pmol of each primer, 1 U Taq DNA polymerase (Fermentas), and 5 µL PCR buffer 10X. Reactions were initiated at 1 cycle at 94° for 3 min, followed by 30 cycles at 94°C for 30 s, 55 for 1 min, 72°C for 1.5 min, and a final elongation step at 72°C for 5 min (24).

Agarose gel electrophoresis

"Powdered agarose (0.8% w/v) was boiled in tris-acetic EDTA (TAE) buffer intermittently until the solution became a clear gel. The agarose solution was allowed to cool to 45^{o}C before 7 µl of ethidium bromide was added. The clear gel solution was poured into the gel tray with the comb in place and allowed to solidify. Thereafter, the gel tray and the comb were removed. The gel was placed into the tank containing TAE buffer. Then, 2µl of the tracking dye (bromophenol blue) was mixed with 1µl of 1.5 kb DNA ladder and loaded into the first well. Thereafter, 20µl of the bromophenol blue with 20µl of the sample was mixed and loaded into other wells. The cover of the tank was carefully placed on it and plugged into the power source to run from a negative to a positive direction, making sure it did not run a distance of far more than ¾ of the gel for approximately 30 minutes. Then, the gel was viewed via the UV transilluminator" (23). A 1.5kb standard DNA molecular weight marker (Gene Mate, UK) was used in the study. (44)

Data analysis

The results were analyzed using descriptive Statistical methods. Graphs were generated with the libraries ggplot2; Rcolorbrewer and pheatmap in R-studio (www.rcoreteam). The dendogram was generated un DendroUPGMA software using the MARI scores of the isolates to generate a similarity matrix for the dendrogram construction. The subsequent tree was visualized in TVBOT (https://www.chiplot.online/tvbot.html).

RESULTS

From a total of 128 clinical samples were collected in this study; 75 (58.6) had the growth of bacteria (table 1). Bacteria isolated were *Escherichia coli* (24), *Klebsiella pneumoniae* (21), *Staphylococcus aureus* (11), *Pseudomonas aeruginosa* (10), *Streptococcuspnuemoniae* (4), *Proteus mirabilis* (2), and *Enterobacter aerogenes* (2), which constituted 18.8%, 16.4%, 8.6%, 7.8%, 3.1%, 2.3%, and 1.6%, respectively (table 2). The distribution of Pseudomonas aeruginosa isolates from the different clinical samples in relation to sex and age is shown in table 3. Age groups 21–30 years, 51–60 years, and 61–70 years had 0.8% *P. aeruginosa* occurrence, respectively, while age groups 11–20 years and 31–40 years had 1.6% Pseudomonas aeruginosa occurrence, respectively.

The resistance pattern of the *P. aeruginosa* isolated from clinical samples to antibiotics showed that all the *P. aeruginosa* isolates identified were resistant to 4 of the antibiotics used namely; ampicillin, cloxacillin, erythromycin and tetracycline respectively, and had 30% resistant to imipenem and meropenem respectively (Figure 1). The multi-drug-resistant *P. aeruginosa* isolates (n=3, 30%) were resistant to carbapenem. The result revealed that one of the *P. aeruginosa* isolates was resistant to seven classes of antibiotics, while three were resistant to six classes. Antibiotic resistance related was investigated using dendroUPGMA and the isolates clustered into 2 clades based on their MARI scores (Figure 2a), both clusters harbored positive PCR positive ESBL isolates. Figure 2b shows a heatmap containing antibiotic resistance profiles of the isolates according to the classes of antibiotics tested, including the bla-OXA50 and bla-PAO positivity rates of the isolates tested. From the heat map majority of the isolates were fully susceptible to carbapenem and quinolonce class of antibiotics while the chephalosporins class showed the highest resistance rates (Figure 2b).Figure 3shows the Agarose gel picturesof the PCR reactions, showing positivebla-PAO bands of 180bpseen in multi-drug-resistant*P. aeruginosa w*hile plate 2 showed the bla-OXA50 bands of 700bpseen in the multi-drug resistant *P. aeruginosa.*

Table 1. Distribution of bacteria growth among clinical samples

Samples	No. of samples	No. of yielded growth	No culturegrowth (%)

	(%)	(%)	
Urine	58(45.3)	36(28.1)	22(17.2)
Wound swab	22(17.2)	11(8.6)	11(8.6)
Sputum	20(15.6)	12(9.4)	8(6.3)
Pus	15(11.7)	7(5.5)	8(6.3)
Ear swabs	8(6.3)	6(4.7)	2(1.6)
Burns	5(3.9)	3(2.3)	2(1.6)
Total	128(100.0)	75(58.6)	53(41.4)

Table 2. Percentage of occurrence of bacterial isolates in the clinical samples

Isolates	n = 128	Number (%) of bacterial isolates
Escherichia coli		24(18.8)
Klebsiellapnuemoniae		21(16.4)
Pseudomonas aeruginosa		10(7.8)
Staphylococcus aureus		11(8.6)
Streptococcus pneumoniae		4(3.1)
Proteus mirabilis		3(2.3)
Enterobacter aerogenes		2(1.6)
Total		75(58.6)

Key: n represents total number of clinical samples, %; represents percentage

Table 3. Distribution of *Pseudomonas aeruginosa* isolated from clinical samples in relation to sex and age.

Sex	Total examined n (%)	Number (%) yielded growth of *Pseudomonas aeruginosa*
Male	55(42.9)	4(3.2)
Female	73(57.1)	6(4.6)
	128(100)	10(7.8)
Age group		

≤10	12(9.4)	0(0.0)
11 – 20	6(4.6)	2(1.6)
21 – 30	34(26.6)	1(0.8)
31 – 40	28(21.9)	2(1.6)
41 – 50	22(17.2)	3(2.3)
51 – 60	18(14.1)	1(0.8)
61 – 70	14(10.9)	1(0.8)
≥ 71	4(3.1)	0(0.0)
Total	128(100)	10(7.8)

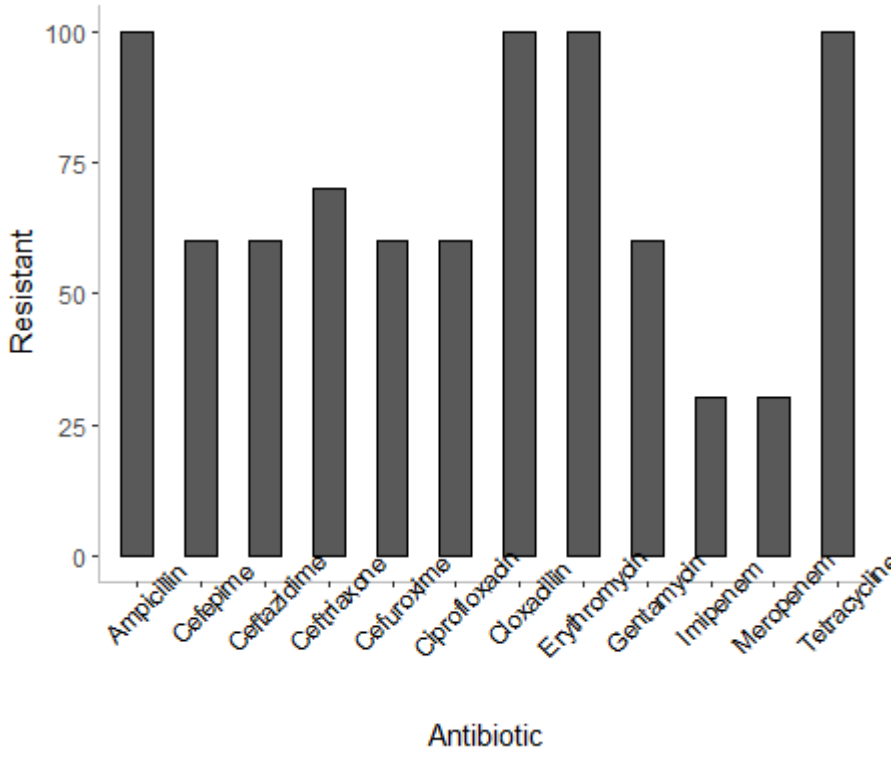
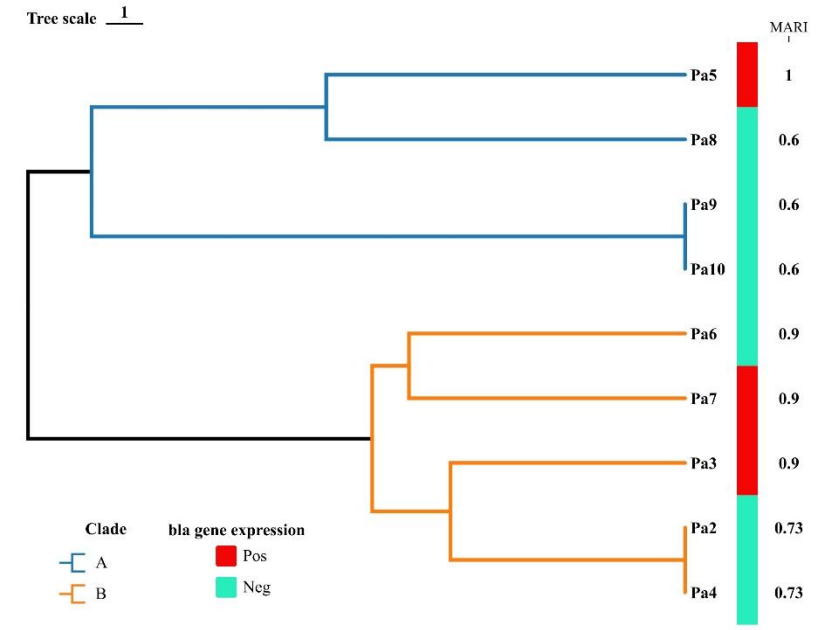


Figure 1. Bar chart showing antibiotic resistance distribution among the various antibiotics tested against *Pseudomonas aeruginosa* isolated from patients in Abeokuta, Nigeria.



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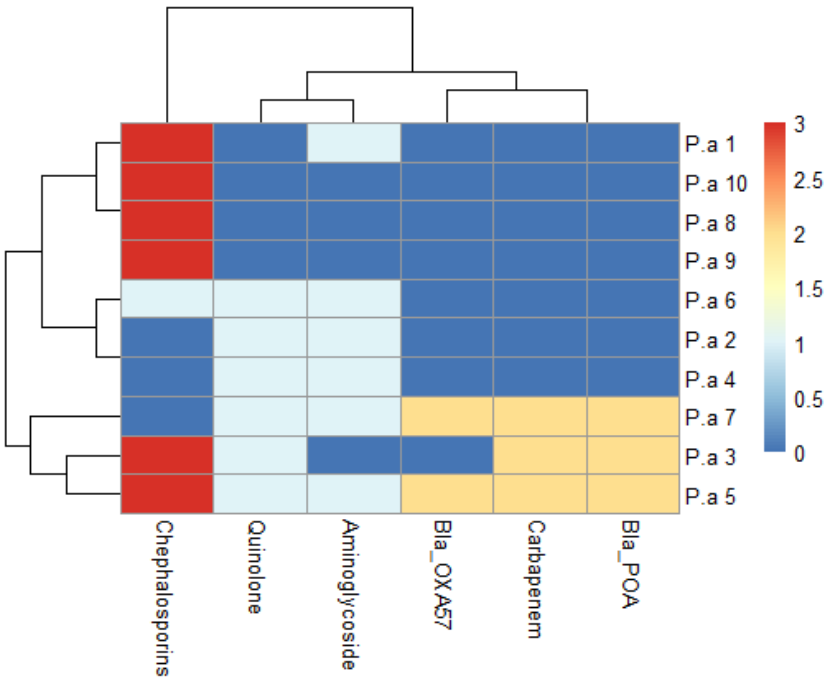
230 Figure 2a



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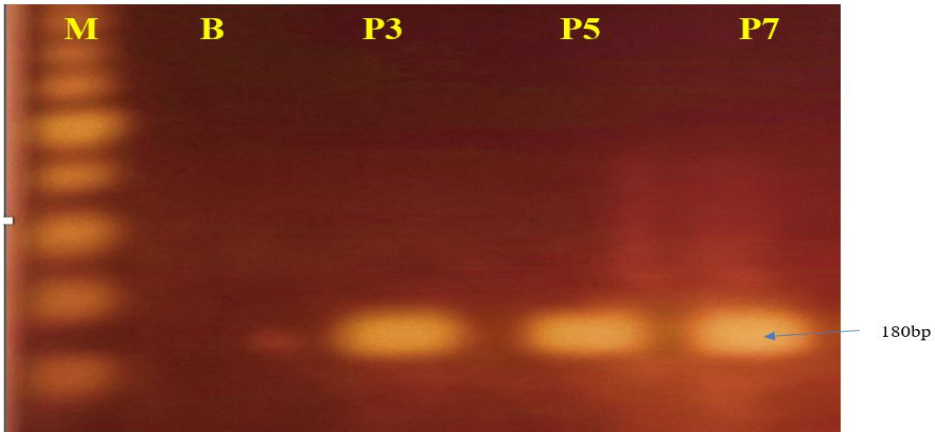
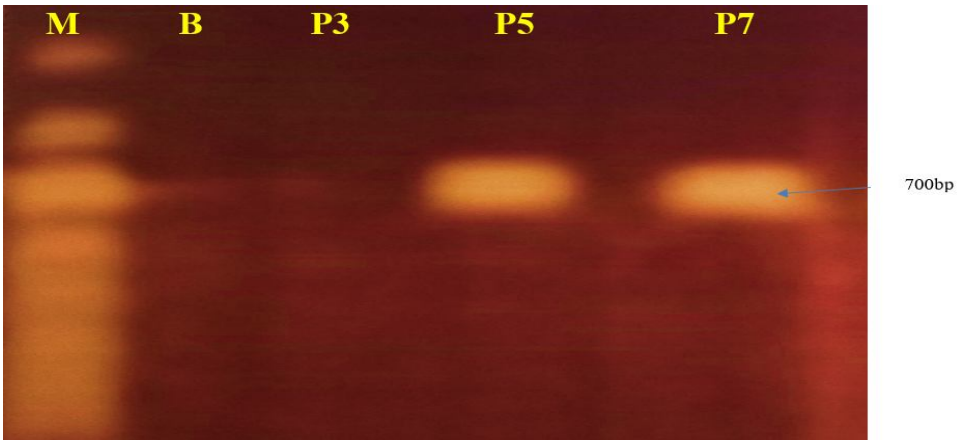
232 Figure 2b

233 Figure 2a. Dendogram showing the antimicrobial based clustering and relatedness, showing,
234 MARI scores and ESBL-PCR positivity. Figure 2b. Heatmap showing resistance profiles of the
235 *Pseudomonas aeruginosa* isolates against the different classes of antibiotics.



Bla-PAO positive



bla-OXA50 positive

Figure 3. Agarose gel pictures of PCR positive samples showing, DNA ladder on the first row, no template control along with PCR positive samples.

DISCUSSION

"*Pseudomonas aeruginosa* is a versatile bacterium that causes a wide range of severe opportunistic infections in patients with serious underlying medical conditions. The prevalence of *Pseudomonas aeruginosa* in this study was 7.8%. *Pseudomonasaeruginosa* is currently one of the most frequent nosocomial pathogens, and infections due to this organism are often difficult to treat due to antibiotic resistance" (25). All the *P. aeruginosa* isolates were completely resistant to ampicillin, cloxacillin, erythromycin, and tetracycline. This finding was in agreement with Akingbade *et al.* (26), who reported that"*P. aeruginosa* was 90% resistant to ampicillin and cloxacillin in south-west Nigeria". According to Juan *et al.*, (27) "*P. aeruginosa* is the third most prevalent bacterium identified from infections contracted in intensive care units and is the main cause of morbidity and death in people with cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), diabetes, and severe kidney and liver failure". "Hospital and community Pseudomonas aeruginosa infection control could suffer major setbacks due to high-level resistance to cell wall biosynthesis inhibitors (particularly Augmentin, ceftazidime and ampicillin) which are most prescribed antibiotics" (28).

"All ten *Pseudomonas aeruginosa* isolates were resistant to three or more classes of antibiotics, and their multidrug patterns cut across all the commonly used drugs prescribed in the clinical setting. This is also consistent with other findings from Egypt" (29). The spread of these antibiotic-resistant *P. aeruginosa* strains is increasing within the hospital environment. This multiple resistance could be attributed to the misuse of antibiotics, which necessitates strict prescription policies to overcome this problem.

"Antibiotic resistance is a major problem observed among most of the *P. aeruginosa* infections in the clinical setting. It can also be seen from these results that *P. aeruginosa* isolates were resistant to most of the commonly used antibiotics. *P. aeruginosa* screened had 60% resistant to gentamycin in this study, and this is similar to the low susceptibility rate reported in two different studies" (32.2% and 33%, respectively) by Samad *et al*. 2017 (30) and Diggle *et al*. 2020 (31). The result is also in agreement with those from Spagnolo *et al*., 2021 (69%) (32), Langendonk*et al*., 2021 (77%) (33), and Shimaa *et al.*, 2023 (73%) (34), but differs from those of Tuon *et al.*, 2022 (31%) (35). "Aminoglycosides are an essential part of the antipseudomonal

chemotherapy used to treat a number of illnesses caused by *P. aeruginosa"* (36, 37).The result also showed that ciprofloxacin inhibited the growth of only 40% of *P. aeruginosa,* and this is in contrast to a 2012 report by Akingbade *et al.* (26) in Abeokuta, Ogun State. Imipenem, a member of the carbapenem class, is the most effective antibiotic (70%) against the strain of *P. aeruginosa* in this study. This is in line with research conducted by Shimaa *et al.* (2023) (34) that found 87.2% of the *P. aeruginosa* isolates susceptible to imipenem. This study revealed the presence of blaPAO and blaOXA-50 among the three strains of MDR-*P. aeruginosa* using the PCR technique. "Many studies have reported the prevalence of blaPAO and blaOXA50 in the *P. aeruginosa* genome" (38). "Due to the ability of *P. aeruginosa* to develop resistance to a wide variety of antibiotics through diverse molecular pathways, the emergence of MDR-*P. aeruginosa* is, in fact, a worldwide health concern.

In the present study, MDR*P. aeruginosa* showed resistance to different antibiotics, such as ampicillin, cloxacillin, erythromycin, tetracycline, cefuroxime, ceftazidime, ceftriaxone, and cefepime. It was also resistant to aminoglycosides (gentamycin) and fluoroquinolones (ciprofloxacin). Recent studies have provided detailed descriptions of each resistance mechanism and contribution to each class of antibiotics" (5, 39). It is known that some strains of *P. aeruginosa* have highly developed and acquired resistance mechanisms that enable them to withstand the majority of antibiotics. The molecular analysis of three multidrug-resistant *P. aeruginosa* shows that two of the *P. aeruginosa* possessed the two types of genes screened for (blaPAO and blaOXA-50). Similar studies have also shown a high incidence of the blaPAO and blaOXA-50 genes among MDR-*P. aeruginosa* (40, 41). It was observed that the two *P. aeruginosa* isolates (p-5 and p-7) that possessed both blaPAO and blaOXA-50 genes were resistant to penicillin, aminoglycosides, tetracyclines macrolides and carbapenems classes respectively. One of this *P. aeruginosa* isolates (P7), was also, sensitive to cephalosporin classes while the other (p-5) was resistant to cephalosporin classes. The *P. aeruginosa* isolate (p-3) that possess only blaPAO gene was resistant to penicillin, aminoglycosides, tetracyclines, cephalosporins, macrolides and carbapenems classes respectively and was sensitive to gentamycin, an aminoglycoside class. Hospital and community antibiotic stewardship need to be strengthened with proper information on combining these antibiotic classes and regulation of

drug prescription, particularly in local outlets (28). The decreased susceptibility of *P. aeruginosa* to commonly used antibiotics has also been shown in different studies (39, 5, 42).

conclusion

In conclusion, this study shows that the three MDR*P. aeruginosa* that have blaPAO and blaOXA-50 beta-lactamases are resistant to penicillin, macrolide, tetracycline, fluoroquinolones, tetracycline, and carbapenems. Newer clinical approaches are needed to curtail the increasing resistance by considering the innovative integrated system in prescription and therapeutic formulation, with combined synergistic mechanism of action (28).

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DECLARATION OF INTERESTS

The authors declare that they have no competing interests regarding this study or decision to publish this manuscript.

ETHICS APPROVAL AND CONSENT

The current study was approved by the ethical committee of Federal Medical Centre, Abeokuta, Nigeria. Informed consent was also given by all participants in the study.

DATA AVAILABILITY STATEMENT

The authors declare that all available data regarding the manuscript is already included within it. Any addition information regarding data of the work will be provided upon request by the corresponding author.

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