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Antibiotic resistance and Virulence Genes of *Pseudomonas aeruginosa* isolates in southwest, Nigeria

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ABSTRACT

Pseudomonas aeruginosa is a ubiquitous bacterium that causes various hospital- acquired and community-acquired infections. It has been reported that the clinical isolates of *P. aeruginosa* are difficult to treat because of their virulence factors and antibiotics resistances. The aim of present study was to screen the antibiotic resistance patterns and the prevalence of virulence factor genes in a set of *Pseudomonas aeruginosa* isolated from Ogbomoso, and to determine whether a correlation exists between the prevalence of virulence factors and antibiotic resistance of *P. aeruginosa.* A total of 100 *P. aeruginosa* isolates were collected from various types of clinical specimens. Antimicrobial susceptibility testing was performed using the Kirby-bauer method. In addition, PCR assays were used for screening four virulence encoding genes (OPRL, LasB, PLCH and ToxA). The results showed that OPRL (79%) and LasB (62%) were the most frequent virulence genes in *P. aeruginosa* strains, followed by PLCH (41%) and ToxA (35%). The highest resistance was detected towards Piperacillin (42%) and Tetracycline (42%). Moderate rate of resistance (12-39%) were detected towards the other antibiotics. The virulent factors identified in this study provide valuable information regarding the prevalence of resistance genes of *P. aeruginosa* isolates in Ogbomoso, Nigeria and their potential impact on treatments that exploit the unique physiology of the pathogen. This will be useful for the health workers to improve infection control measures and to establish a surveillance system.

Keywords: antibiotic resistance, virulence genes, *Pseudomonas Aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous gram-negative bacillus which is responsible for different hospital-acquired and community-acquired infections. This bacterium is considered an opportunistic pathogen that affects the health of immunocompromised individuals such as those with diabetes, cancer, cystic fibrosis, advanced HIV infections, severely burned patients and those that underwent major surgeries [1]. *Pseudomonas aeruginosa* is responsible for several nosocomial infections like pneumonia, urinary tract infections, surgical site infections, and some of the community-acquired infections such as otitis externa, ulcerative keratitis, and soft tissue infections. P. aeruginosa is associated with high morbidity and mortality and it has been reported as an acute infection in over 70% of cases [2]. As a pathogen, *Pseudomonas aeruginosa* is of increasing clinical importance because of its innate resistance to multiple agents and its ability to develop high-level multidrug resistance (MDR) due to the presence of several virulence factors encoded in its genome [3]. One of these virulence factors that play

a main role in tissue lysis and bacterial invasion is exotoxin A (exoA). The hemolysin phospholipase H (plcH) act to destroy lipids and lecithin contributing to tissue invasion. *P. aeruginosa* also produces exoenzyme S (exoS), which is the cytotoxin responsible for damage to many types of host cells and elastase B (lasB) that play an important role during the acute infection [3-4]. Some strains produce alginate that forms the matrix of the biofilm which protects bacteria from the host defence during the chronic infection. The GDP-mannose 6-dehydrogenase (algD) is one of three proteins that are implicated in the production of alginate [5-6]. These toxins are thought to promote the organism, diffusion in the site of infection and the organism invade to host immune system and inhibits DNA synthesis, so leads to host cell damage and death [7]. Several studies have shown that this innate resistance is directly associated with the expression of bacterial efflux pumps and porins such as the Resistance-Nodulation-cell-Division (RND)-type efflux pump, MexAB-OprM, MexC–MexD–OprJ, MexE–MexF–OprN, and MexXY-OprM. [8]. These important RND-type efflux pumps are constitutively expressed in wild-type cells and are responsible for the intrinsic resistance to most antimicrobial agents [9]. Under iron-limiting conditions, *P. aeruginosa* produces two main siderophores, pyoverdine and pyochelin, which scavenge iron from host proteins, contributing to its virulence [10]. The ferri-pyoverdine complex uptake is carried out by TonB-dependent receptors with the help of the transporter FpvB, resulting in its internalization into the periplasm. In addition to its molecular function, the pyoverdine receptor genes have also been used to genotype several strains of *P. aeruginosa.* Therefore, the characterization of B-lactamases from drug-resistant *P. aeruginosa* can greatly contribute to the understanding of mechanisms of molecular pathogenesis, antibiotic resistance and virulence in bacteria [11].

MATERIALS AND METHODOLOGY

MATERIALS

The materials used include disposable protective latex gloves, cotton wool, immersion oil, normal saline, glass slides, coverslips, Pasteur pipettes and wire loop.

STUDY AREA

The study areas are UNIOSUN Teaching Hospital, Osogbo, Osun State; OAU Teaching Hospital, Ile-Ife, Osun State; University College Hospital, Ibadan, Oyo State; and LAUTECH Teaching Hospital, Ogbomoso, Osun State.

ETHICAL APPROVAL

Ethical approval was obtained from Ethical Review committee of UNIOSUN Teaching Hospital, Osogbo, Osun state.

INCLUSION CRITERIA

All *Pseudomonas aeruginosa* isolates obtained from clinical samples from patients of all age-group, gender and ethnicity were included.

EXCLUSION CRITERIA

Pseudomonas aeruginosa isolates obtained from non-clinical samples (such as animal samples, water samples, food samples, etc. were excluded.

LABORATORY ANALYSIS

Isolation Identification of *Pseudomonas Aeruginosa*

From September 2021 to December 2021, one hundred *P. aeruginosa* isolates from wound swab, urine, stool, sputum, blood cultures, cerebrospinal fluid, and catheter tip samples obtained from different patients were collected from the Medical microbiology laboratories of four hospitals located at Ibadan, Ogbomoso, Osogbo and Ile-Ife.

Preliminary identification of bacteria was based on colony characteristics of the organisms including beta hemolysis on blood agar, non-lactose fermentation and pigment production (greenish yellow and bluish green pigments) on Mac-Conkey agar. The isolates that showed these characteristics were then sub-cultured onto blood agar to obtain a pure culture.

Gram Staining and Microscopy

Gram staining was performed on colonies from subcultures for the identification of their gram reaction. Specimens were smeared onto clean grease-free glass slides, air dried, heat fixed and gram stained. The stained slides were identified as gram-negative if they did not retain the purple stain of crystal violet and were counter-stained pink by safranin.

Biochemical Tests

Oxidase Test

A colony of organisms was smeared on a filter paper soaked with a drop of oxidase reagent. It was observed for colour change. A purple colouration indicated an oxidase producing organism (*Pseudomonas aeruginosa*)

Catalase Test

A drop of Hydrogen peroxide was placed on a glass slide, and a colony of organisms was emulsified in it and observed for gas bubble production. The production of gas bubbles signified the presence of catalase producing bacteria (*Pseudomonas aeruginosa*).

ANTIMICROBIAL SUSCEPTIBILITY TESTS

Disc Diffusion

The susceptibility testing was determined by the disc diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations. Inhibition zone diameters were evaluated according to EUCAST guidelines [12]. The antimicrobial agents assayed in this study include Ceftazidime (CAZ), Meropenem (MEM), Imipenem (IPM), Cefoxitin (FOX), Cefepime (FEP), Cefotaxime (CT),aztreonam (ATM), ticarcillin (TIC), Ciprofloxacin (CIP), Amikacin (AK), Ofloxacin (OFX), Cefalexin (CN), Tetracycline (TE), Tobramycin (TOB), Piperacillin (PRL), Ceftriaxone (CRO), Trimethoprim-sulfamethoxazole (SXT), Levofloxacin (LEV), Ticarcillinclavulanic acid (TIM), Piperacillintazobactam (TZP). The minimum inhibitory concentrations (MICs) of imipenem were determined for all strains that displayed resistance or intermediate resistance to imipenem, by using the broth microdilution method as described elsewhere. The results were interpreted based on the EUCAST breakpoints [12]. The multidrug-resistant (MDR) phenotypes are defined as resistance to at least three or more classes of antimicrobial agents. *P. aeruginosa* ATCC 27853 was employed as a control strain.

GENOTYPIC ANALYSIS

DNA Extraction for *Pseudomonas aeruginosa*

The isolates were plated out on Mueller Hinton agar and incubated at 37˚C for 18 to 24 hours. The DNA of the isolates were extracted by suspending 4-5 bacteria colonies in 200 µl of molecular grade water in Eppendorf tubes appropriately labelled. The mixture were vortexed vigorously. The cells were boiled at 100°c for 10 minutes and were cooled rapidly on ice for 30 minutes. The cell lysate were centrifuged briefly at high speed (13, 400 rpm for 3 min), and the supernatants containing the genomic DNA were transferred into fresh sterile Eppendorf tubes. The extracted DNAs were stored at -21˚C until required for PCR.

PCR Procedure for Gene Amplification

A 20µl reaction containing 4µl of 5X ready to load mastermix, 0.5µl of forward primer, 0.5µl of reverse primer, 2 µl of DNA lysate and 13µl of nuclease-free water was used for PCR. Amplifications were subjected to initial denaturation at 95^{o}C for 5min, followed by 35 cycles of denaturation at 95^{o}C for 1 min, annealing at 55^{o}C (OPRL), 56^{o}C (Tox A), 56^{o}C (PLCH), and 56^{o}C (LasB) for 1 min respectively, extension at 72^{o}C for 1 min and final extension procedure was carried out at 72^{o}C for 10min [13].

Gel Electrophoresis

After the amplification, PCR products were resolved on l.5% agarose gel prepared by dissolving 1.5g of agarose powder in 100 ml of 1X Tris-borate-EDTA (TBE) buffer solution inside a clean conical flask. The 1.5% 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 ^{0}C after which 0.5 µl of ethidium bromide was then 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, 5µl of the amplicon was mixed with 1µl of loading buffer and the mixture was loaded to the wells of the agarose gel. The power supply was adjusted to 100 volts for 25 minutes. For each run, a 100 base-pair molecular 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 a gene gel bioimaging system. The PCR product was then analyzed.

RESULTS

Table 1: Antibiotic susceptibility pattern of the isolated *P. aeruginosa* in the study population n (%)

ANTIBIOTICS	SENSITIVE	INTERMEDIATE	RESISTANT
Ceftazidime (CAZ)	54 (54)	10 (10)	36 (36)
Meropenem (MEM)	56 (56)	10 (10)	34 (34)
Imipenem (IPM)	58 (58)	7 (7)	35(35)
Cefoxitin (FOX)	51 (51)	11 (11)	38 (38)
Cefipime (FEP)	53 (53)	9 (9)	38 (38)
Cefotetan (CT)	88 (88)	0 (0)	12 (12)
Aztreonam (ATM)	67(67)	8 (8)	25 (25)
Ticarcillin (TIC)	50 (50)	11 (11)	39 (39)
Ciprofloxacin (CIP)	64 (64)	8(8)	28(28)
Amikacin (AK)	62 (62)	9 (9)	29 (29)
Ofloxacin (OFX)	62 (62)	9 (9)	29 (29)
Gentamicin (CN)	63 (63)	9(9)	28 (28)
Tetracycline (TE)	48 (48)	10 (10)	42 (42)
Tobramycin (TOB)	57 (57)	7 (7)	36 (36)
Piperacillin (PRL)	49 (49)	9 (9)	42 (42)
Ceftriaxone (CRO)	60 (60)	7 (7)	33 (33)
Trimethoprim/ Sulfamethoxazole (SXT)	65 (65)	7 (7)	28 (28)
Levofloxacin (LEV)	53 (53)	8 (8)	39 (39)
Ticarcilline+ Clavulanic acid (TIM)	65 (65)	7 (7)	28 (28)
Piperacillin/Tazobactam TZP	70 (70)	10 (10)	20(20)

Table 1 shows the rate of sensitivity, intermediary and resistance of 100 different *P. aeruginosa* isolates to each of the twenty (20) antibiotics used. This result shows that a higher percentage of the organisms are sensitive to the antibiotics used with a range of 48-88% sensitivity and 12-42% resistance. In the susceptibility tests, 36 (36%) strains showed resistance to Ceftazidime, 34 (34%) strains showed resistance to Meropenem, 35 (35%) strains showed resistance to Imipenem, 38 (38%) strains showed resistance to Cefoxitin, 38 (38%) strains showed resistance to Cefipime, 12 (12%) strains showed resistance to Cefotetan, 25 (25%) strains showed resistance to Aztreonam, 39 (39%) strains showed resistance to Ticarcillin, 28 (28%) strains showed resistance to Ciprofloxacin, 29 (29%) strains showed resistance to Amikacin, 29 (29%) strains showed resistance to Ofloxacin, 28 (28%) strains showed resistance to Gentamicin, 42 (42%) strains showed resistance to Tetracycline, 36 (36%) strains showed resistance to Tobramycin, 42 (42%) strains showed resistance to Piperacillin, 33 (33%) strains showed resistance to Ceftriaxone, 28 (28%) strains showed resistance to Trimethoprim/Sulfamethoxazole, 39 (39%) strains showed resistance to Levofloxacin, 28 (28%) strains showed resistance to Ticarcilline+ Clavulanic acid, and 20 (20%) strains showed resistance to Piperacillin/Tazobactam.

Table 2: Showing the distribution of the virulence factors in the samples

	Virulence Genes in *P. aeruginosa* isolates (n=100)			
Sample	OprL	PLCH	LasB	ToxA
Catheter tip	5	2	3	1
Ear swab	18	13	14	9
Eye swab	7	4	6	3
Sputum	12	8	9	5
Urine	9	2	8	3
Vaginal swab	17	8	12	8
Wound swab	11	4	10	6
Total	79	41	62	35

From Table 2, the results highlighted that OPRL (79%) and LasB (62%) were the most frequent virulence genes in *P. aeruginosa* strains, followed by PLCH (41%), while the least commonly detected virulence factor gene was ToxA (35%). Showing the distribution of the virulence factors in the samples, the results showed that, isolates from ear swabs had the highest frequency of virulence factors.

Table 3: showing the association between the virulence factors and their resistance to antibiotics

Virulence factor	OprL	PLCH	LasB	ToxA
Antibiotics				
CAT.	29	16	24	18
p-value	.775	.599	.471	.018
MEM.	28	11	21	9
p-value	.555	.207	.972	.199
IPM.	24	12	16	10
p-value	.060	.316	.014	.323
FOX.	34	18	25	19
p-value	.108	.402	.729	.021
FEP.	33	16	26	15
p-value	.132	.860	.300	.463
CT	7	7	9	3
p-value	.061	.193	.323	.439
ATM.	18	10	13	8
p-value	.321	.907	.234	.717
TIC.	32	20	24	15
p-value	.549	.095	.939	.562
CIP.	23	13	18	11
p-value	.630	.491	.769	.575
AK.	22	11	15	5
p-value	.787	.635	.313	.028
OFX.	22	9	16	5
p-value	.622	.195	.369	.017
CN.	23	10	18	9
p-value	.630	.503	.769	.709
TE.	31	18	24	15
p-value	.141	.879	.268	.983
TOB.	25	15	19	13
p-value	.078	.919	.154	.861
PRL.	33	24	27	17
p-value	.929	.005	.689	.329
CRO.	28	14	20	11
p-value	.314	.839	.840	.806
SXT.	24	15	18	13
p-value	.304	.111	.769	.135
LEV.	29	12	22	9
p-value	.362	.096	.357	.046
TIM	22	12	17	9
p-value	.046	.814	.869	.709
TZP	16	9	13	8
p-value	.902	.684	.757	.600

N.B: *p-value* ≤ 0.05 is taken to be statistically significant.

Figure 1: antibiotic resistance pattern of the isolates based on their virulence factors

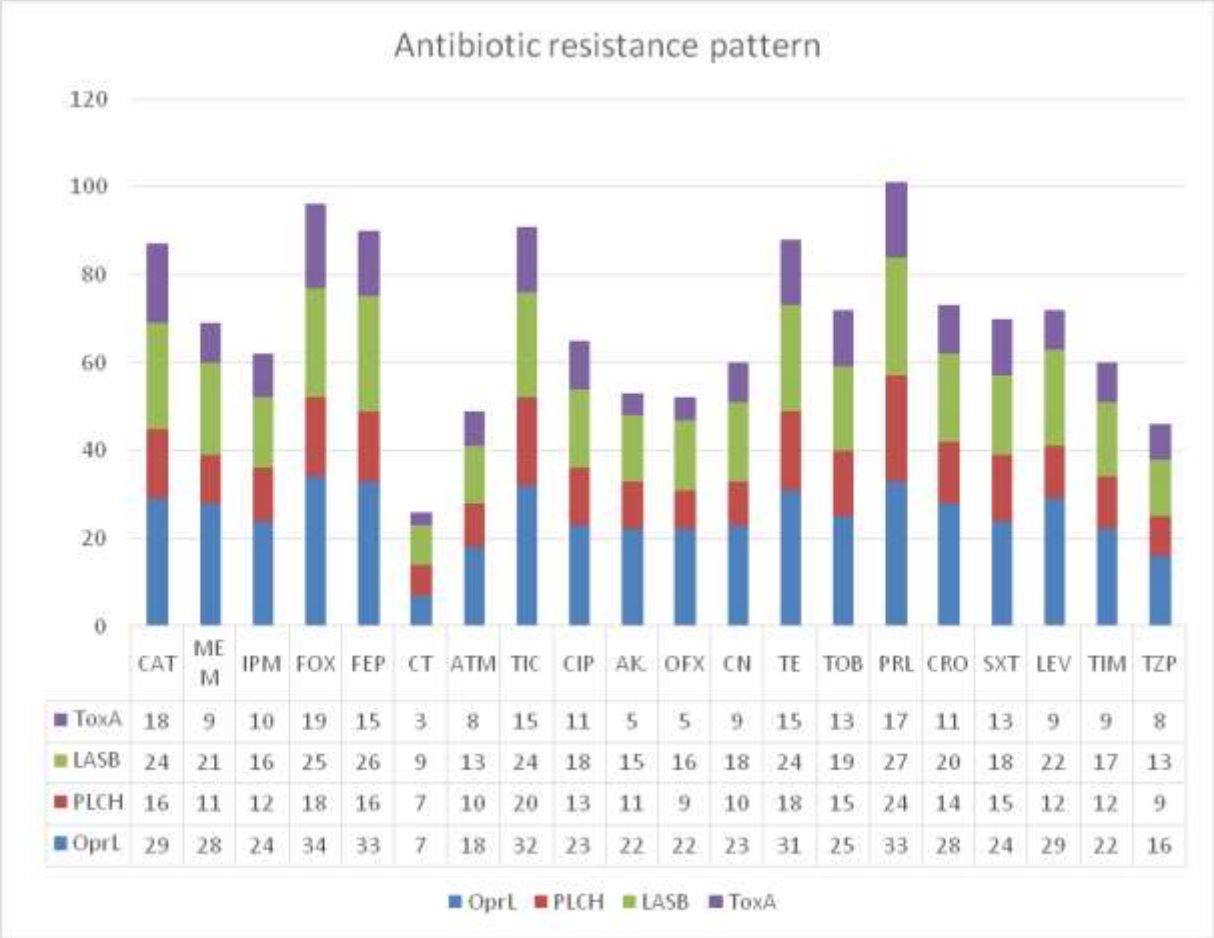
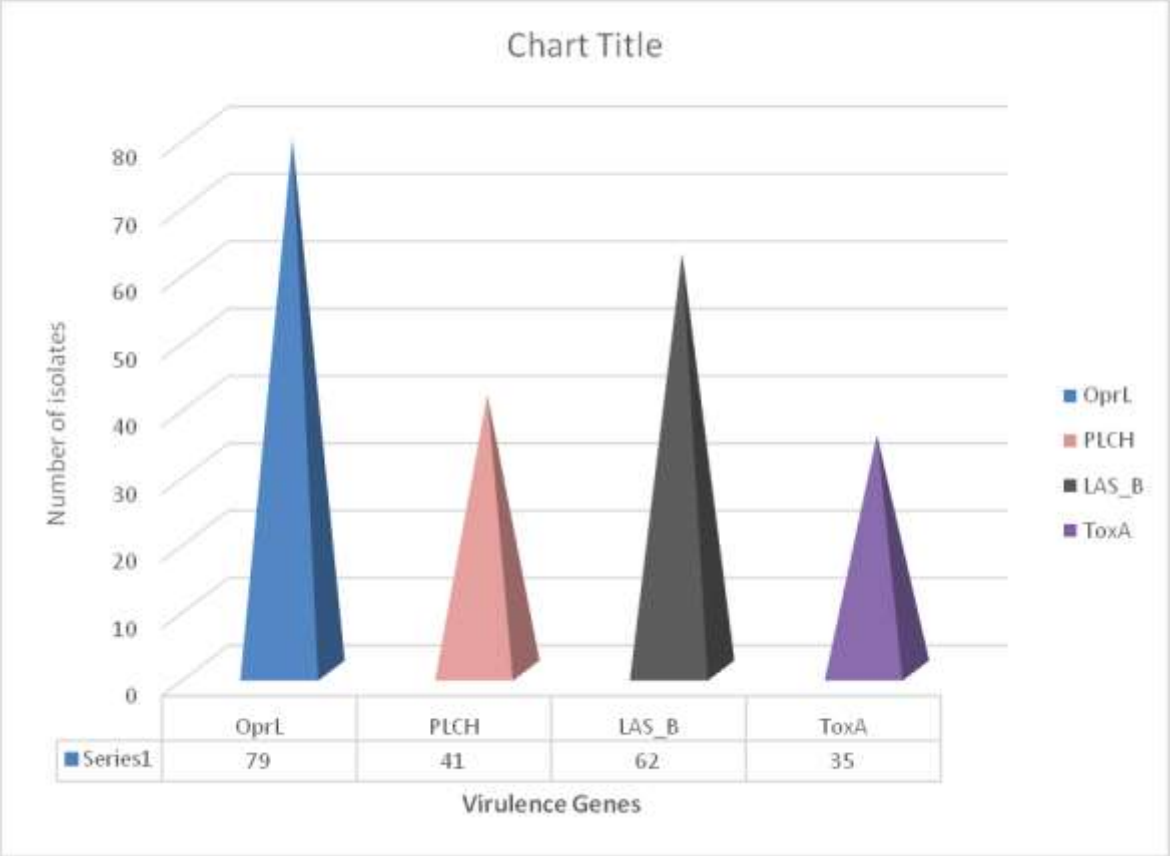
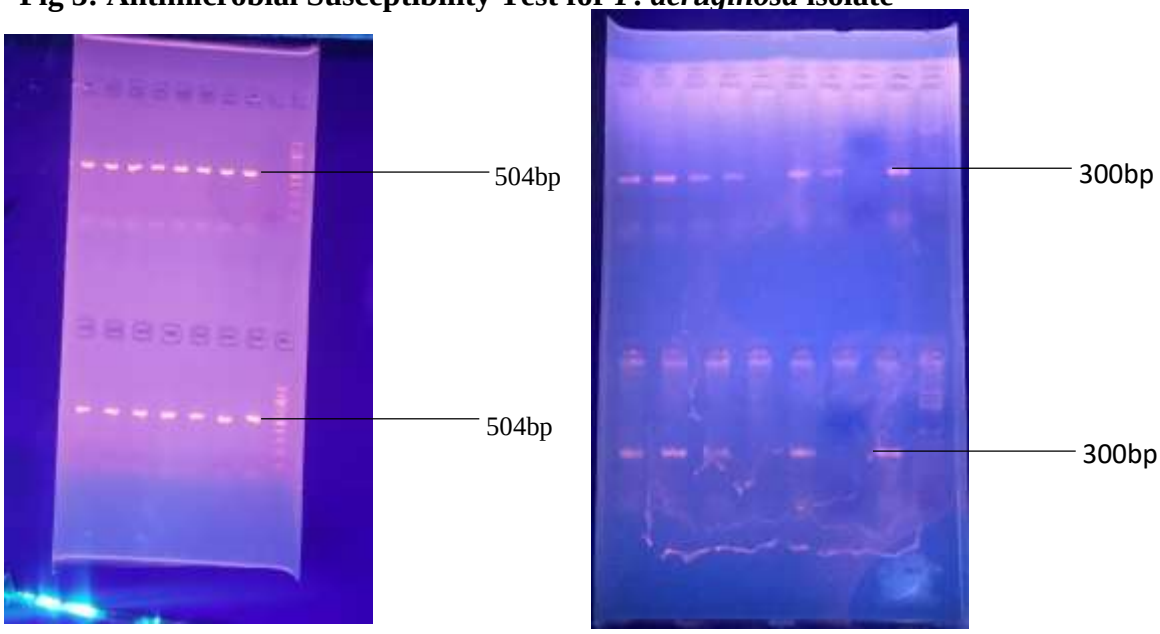


Figure 2: Virulence factors in *P. aeruginosa*



From Figure 2, the results showed that OPRL (79%) and LasB (62%) were the most frequent virulence genes in *P. aeruginosa* strains, followed by PLCH (41%), while the least commonly detected virulence factor gene was ToxA (35%).

Fig 3: Antimicrobial Susceptibility Test for *P. aeruginosa* isolate



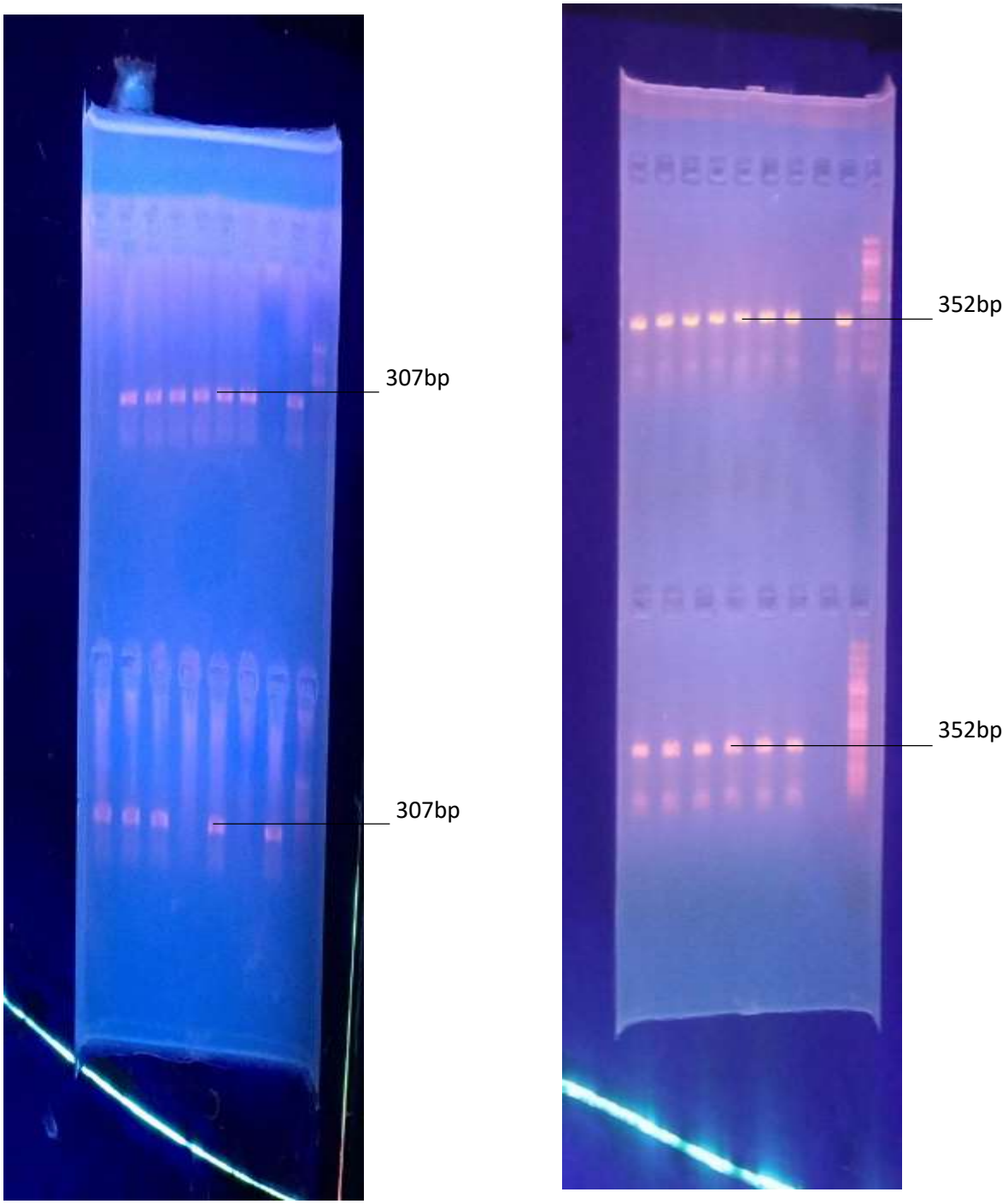


Fig. 4 Gel Electrophoresis of PCR products of the virulence genes among *Pseudomonas aeruginosa* isolates.

(1) Amplification of the OPRL gene, (2) amplification of the LasB gene, (3) amplification of the PLCH gene and (4) amplification of the ToxA gene. L=DNA ladder, C=Positive control, N=Negative control.

DISCUSSION

Pseudomonas aeruginosa is widely known to significantly cause nosocomial infections with high mortality and morbidity rate, especially in immunocompromised patients [14]. Findings from this study have critically highlighted important potential clinical sources of this bacterium which include catheter tip (5%), ear swab (24%), eye swab (8%), sputum (14%), urine (11%), vaginal swab (21%) and wound swab (17%). Earlier reports from Nigeria on the isolation rates of this organism from clinical samples include those of Odunsanya [15] who reported 4.6% for urine and 16.3% for wounds, and from Ogbolu *et al.* [16] who reported 41.9% and 39.35% for ear and wound swab respectively. However, from this study majority of the isolate used were taken from ear swabs as it accounts for 24% of the total isolate.

It is interesting to note that Amikacin which shares the same aminoglycoside class as gentamicin is also very effective as 62% of the isolates were found to be susceptible to it. However, this is lesser than the 99.4% reported by Elmouaden *et al.* [17] and that of Mohammadzadeh *et al.* [18]. In the present study, 50% of the isolates were sensitive to ticarcillin as opposed to the 46% reported in Sarwat *et al.* [19] which were resistant.

It was found that the rates of antibiotic resistance of P. aeruginosa were (25%) to aztreonam, (28%) to ciprofloxacin, and (36%) to ceftazidime, whereas Badamchi *et al.* [20] reported that (27.1%) of P. aeruginosa were resistant to ciprofloxacin and (25.9%) to ceftazidime. Several surveys from developed and developing countries admit the direct link between irrational antibiotics use and the emergence of resistant strains. To reduce this problem, it is important to implement infection control measures such as good hand hygiene and proper use of antimicrobial agents [20].

Furthermore, our results revealed that the majority of the isolates are susceptible to Carbapenems, (58%) to Imipenem and (56%) to Meropenem. These findings are not in agreement with the previous result of a study done by Gierhart *et al.* [21] in which all strains exhibited significant decreases in susceptibility to imipenem. However, it is in accordance with that of Kireçci *et al.* [22] in which the majority of the isolates were susceptible to imipenem and meropenem.

As opposed to the study of Li *et al.* [23] and Isichei-Ukah and Enabulele [24] the findings of this study shows that majority of the isolates were susceptible to Ofloxacin (62%), Levofloxacin (53%), Tobramycin (57%), Trimethoprim/ Sulfamethoxazole (65%), Piperacillin/Tazobactam (70), Ticarcilline+ Clavulanic (65%).

In accounting for the susceptibility of the isolates to cephalosporins, they were found to be susceptible to Cefotetan (88%), Ceftriaxone (60%), Ceftazidime (54%), Cefoxitin (51%), and Cefipime (53%). This does not agree with the study conducted by Jazani *et al.* [25] in which there was 75.4% resistance to Cefepime and that of Prakash *et al.* [26] who reported 65.26% resistance. This could have resulted from adherence to the control programs organized to curb antibiotic resistance that must have included the proper usage ofantibiotics. Moreover, the high sensitivity of the isolates to Ceftazidime (54%) is congruent with the study of Oladipo *et al* [27] who reported 76% sensitivity, Kechrid and Hassen [28] who found sensitivity to be 97%.

PCR assays were used for screening four virulence encoding genes present in a variable amount in the isolates engaged for the study, the genes and their level of occurrence are as follows; *Oprl* (79%), *plcH* (41%), *lasB* (62%) and *ToxA* (35%). Of all the four genes identified in the isolates, Oprl occurred the most as it was identified in 79% of the isolates.

The *LasB* gene which is one of the most important proteases of *P.aeruginosa* has a 62% occurrence in the isolates used for this study, this is lesser than the 98.7% occurrence reported in the study by Elmouaden *et al.* [17].

The 41% occurrence of the plcH gene in the isolates is dissimilar to that reported by Elmonaden *et al.* [17] where almost all (96.1%) harboured the plcH gene and that

of Lanotte *et al.,* 2004 where all strains possessed the gene. Although the ToxA gene has the lowest (35%) level of occurrence in the isolates, it, however, sponsors the majority of the statistically significant resistance of the organism to antibiotics such as in resistance to CAZ (p=0.018), FOX (p=0.021), OFX (p=0.017), and AK (p=0.028). Its occurrence in the present study is opposed to the 69.4% reported in the study of Badamchi *et al.*[20].

The divergences in the distribution of virulence factor genes in the different populations might be due to the probability that some *P. aeruginosa* strains are better adapted to the conditions found in infectious sites that may be returned to the diverse geographical and environmental sources. The prevalence of *P. aeruginosa* and its virulence genes depends on various causes consisting of the nature of places, degree of contamination and type, immune status of individual patients, and virulence of strains.

The main advantage of this investigation was the collection of a set of strains isolated from various samples to determine the antibiotic susceptibility patterns and the prevalence of virulence factor genes, and although most previous studies have focused separately on virulence or resistance, this current study assessed the potential relationship between the distribution of virulence factor genes and antibiotic resistance profiles *in P. aeruginosa* strains. This survey provides data about phenotypic and genotypic characteristics of *P. aeruginosa* that emerged in Southwest, Nigeria, which could be useful for the health workers to improve infection control measures and establish a surveillance system.

CONCLUSION

The present study provides an insight into antimicrobial resistance and virulence factors of *P. aeruginosa* isolates in Southwest, Nigeria. Our finding highlighted a moderate rate of resistance to antibiotics. The virulent factors identified in this study provide valuable information regarding the prevalence of resistance genes and their potential impact on treatments that exploit the unique physiology of the pathogen.

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