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Full Length Research Article

Antibiotic Resistance and Transfer of Extended Spectrum Beta Lactamase (ESBL) Resistant Plasmids in *Pseudomonas aeruginosa* Isolates from Hospitalized Patients

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ABSTRACT

The study was undertaken to determine antibiotic resistance, ESBL production and transfer of resistant plasmids of *Pseudomonas aeruginosa*. Clinical samples of urine, wound and blood were screened for *Pseudomonas aeruginosa* following standard microbiological procedures. Isolates of *Pseudomonas aeruginosa* were identified in a total of 110 (18.3%) samples with higher prevalence observed in wound (43.6%) and blood (40.0%) samples. Identified isolates were profiled for antimicrobial susceptibility to a total of 10 antibiotics. Plasmid DNA extraction was carried out by the alkaline lysis method. Double disc synergy test (phenotypic) and PCR (genotypic) techniques were used to determine ESBL production from selected organisms while transfer of resistant genes was assessed by conjugation using broth mating procedures. Results showed high rates of resistance among organisms tested using antipseudomonal drugs. Seventy-seven (70.0%) of the isolates were multi drug resistant (MDR), out of which 26 (33.8%) and 4 (15.4%) produced ESBL by phenotypic and genotypic methods, respectively. Plasmid profile analysis revealed that all the 77 MDR isolates harboured plasmids of > I.5 kb. Four ESBL producing isolates selected could transfer their resistance plasmid to a recipient *E. coli* strain K-12. The therapeutic challenge observed calls for an effective antimicrobial stewardship program.

Keywords: Multidrug-Resistance (MDR), ESBL production, *Pseudomonas aeruginosa,* antipseudomonal drugs

INTRODUCTION

Multidrug resistance is emerging globally and is of great concern especially in infections caused by *Pseudomonas aeruginosa*. *P. aeruginosa* is an opportunistic pathogen that thrives best in moist settings including health care settings (Lister *et al*., 2009). *P. aeruginosa* rarely colonizes healthy individuals but frequently found to spread among hospitalized patients (>50% colonization rate) and has been implicated in most hospital acquired infections (Arora *et al*., 2011). The organism has innate resistance to many antibiotics including β-lactams, anti-pseudomonal Ceftazidime, aminoglycosides and ciprofloxacin (Dundar and Otkun, 2010; Potron *et al*., 2015), making infections they cause difficult and expensive to treat. Similarly, increasing resistance to β-lactams such as third and fourth- generation cephalosporins has become a serious global clinical problem due to the production of beta-lactamases. *Pseudomonas aeruginosa* is known to produce extended spectrum beta-lactamases (ESBLs) (Memish *et al.,* 2012) and now frequently isolated (Potron *et al*., 2015.)

ESBLs are plasmid mediated enzymes, capable of hydrolyzing and inactivating a wide variety of beta-lactam antibiotics including oxyimino-β-lactams (third- and fourth- generation of cephalosporins) and aztreonam, and are inhibited by β-lactamase inhibitors, such as clavulanic acid (Paterson and Bonomo, 2005). These features are employed in the phenotypic tests used for the detection of ESBL-producing strains. The ESBL-type enzymes are categorized into two Ambler classes, A and D. The plasmid mediated antibiotic resistance facilitates the ease of spread between species. Being plasmid mediated, ESBLs are often co-resistant to aminoglycosides, sulfonamides and quinolones. Therefore, infections caused by such strains may be difficult to treat due to limited therapeutic option

The increasing resistance to beta-lactam antibiotics has led to an increased focus by

researchers to determine the mechanisms of resistance. Until recently only a few studies have been carried out to detect such mechanism in Nigeria. The present study was conducted with an aim to detect the prevalence of ESBL-producing *Pseudomonas aeruginosa* isolates, their susceptibility to selected antibiotics and transfer of resistance from clinical samples of two government funded hospitals in Delta State, Nigeria.

MATERIALS AND METHODS

Study Site and Collection of Samples

The study examined 600 samples collected from wound, urine and blood of hospitalized patients in 2 general hospitals at Warri and Agbor located in Delta State, Nigeria. One hundred of each sample type was collected based on the principle of one sample type from one patient. The samples were collected in 2014 after obtaining approval from the post graduate thesis research committee Delta State University and the hospitals ethical committee. Written or verbal consent from the patients or their guardians was obtained before samples were collected from the patients. The samples were collected aseptically using sterile swab stick and universal bottles following standard microbiological guidelines (Collee and Marr, 2008).

Culture and Identification of *Pseudomonas aeruginosa*

Following aseptic collection, urine and wound swabs were cultured on Blood and MacConkey agar media. For blood culture, samples were first inoculated into tryptic soya broth to enhance growth and incubated aerobically at 37 ^{o}C for 24 hrs. The broths were then sub cultured on 5% sheep blood agar and MacConkey agar. Cultures showing signs of growth were followed-up by examining the broth daily and conducting a final sub-culture at the end of the 7th day or at the appearance of turbid culture. The plates were incubated aerobically overnight at 37 ^{o}C and observed for bacteria growth. *P. aeruginosa* were identified by their colony characteristics, pigment production, staining characters, motility and other relevant biochemical tests following standard methods of identification (Palleroni, 1984).

Antimicrobial Susceptibility Testing

The antibiotic susceptibility of *P. aeruginosa* to selected antibiotics was determined using Mueller-Hinton agar (MHA) plates (Hi media, India) and by the disc diffusion method according to the CLSI guidelines (CLSI, 2012). Multi-antimicrobial discs containing the following antibiotics and their concentration were used; amoxicillin-clavulanic acid (A-30 µg), ceftazidime (CZ-30 µg), cefotaxime (CF-30µg), cefuroxime (CR-30 µg), cefixime (CX-5 µg), gentamicin (G-10 µg), ciprofloxacin (C-5 µg), ofloxacin (O-5 µg), trimethoprim-sulfamethoxazole (S-25 µg), and nitrofurantoin (N-300 µg). Zones of inhibition around each disc were measured and recorded as sensitive or resistant. Multi drug resistance (MDR) among the isolates defined as resistance in ≥ 3 antibiotics were selected.

Phenotypic Detection of ESBL Production

Detection of ESBL production by MDR *Pseudomonas aeruginosa strains* was performed on MHA plates by Double disc synergy test (DDST) as described by Jarlier *et al*. (1988). Mueller Hinton agar (Hi Media, India) was inoculated with 0.5 McFarland standard inoculum of the MDR *Pseudomonas aeruginosa* isolate using sterile cotton swab. An amoxicillin-clavulanic acid disc was placed in the centre of the plate and the test antibiotics (ceftazidime and cefotaxime) were placed at 20 mm distance (center to center) from the amoxicillin-clavulanic acid disc. The plates were incubated at 37 ^{o}C for 24hrs. Enhancement of zone of inhibition towards amoxicillin -clavulanic acid was interpreted as ESBL producer.

Plasmid DNA Isolation and Agarose Gel Electrophoresis

Plasmid DNA extraction was carried out by the alkaline lysis method of Birnboin and Dolly (1979). The extracted DNA were resolved on 1.5% agarose gel. Electrophoresis was conducted on a horizontal gel apparatus for 45 mins at 90V using 0.5% Tris – Borate – EDTA (TBE) buffer (pH.8.3). The molecular size of the

plasmids was estimated by comparing with standard plasmids of known molecular weight in the gel.

Molecular Detection of ESBL Genes

The ESBL phenotypic positive isolates were genotyped by performing PCR using specific primers for the detection of ^{bla} CTX-M, ^{bla} TEM, and ^{bla} SHV genes as shown in Table 1. Here 5 µl DNA was used in 25 µl reaction mixture containing 2.5U of *Taq* polymerase (Thermo Scientific, Germany), 200 µm each of dATP, dGTP, dTTP and dCTP, 0.2 µm each primer, 1.5 mM $MgCl_2$. PCR amplification conditions were initial denaturation at 94 ^{o}C for 10 minutes followed by 35 cycles of denaturation for 30 s at 94 ^{o}C, 1 minute of annealing at 56 ^{o}C, extension at 72 ^{o}C for 1 minute and the final extension was done at 72 ^{o}C for 10 minutes. The PCR amplification products were resolved in 1.5% agarose gel. Staining of the gel was done with ethidium bromide in a TEA buffer. At 100V for 60 min and visualized under UV transilluminator (Alliance 4.7).

Transfer of Antibiotic Resistance

Transfer of resistance by conjugation using broth mating procedure as described by Ahmad *et al.* (1994) was performed. All MDR *P. aeruginosa* with ESBL-positive genotype and plasmid free *E. coli* K-12 showing sensitivity to all tested antibiotics in this study were used as donor and recipient cells, respectively

Table 1: Primer sequence for detection of ESBL genes in *P. aeruginosa* clinical isolates

GENES	PRIMER SEQUENCE (5→3)	AMPLICON SIZE (BP)	REFERENCE
CTX-M 2-F	ATGATGACTCAGAGCATTCG	865	-
CTX-M 2- R	TGGGTTACGATTTTCGCCGC	-	Nedjai *et al*., 2013
SHV- F	TCAGCGAAAAACACCTTG	471	-
SHV-R	TCCCGCAGATAAATCACC	-	Lal *et al*.,2007
TEM-F	CTTCCTGTTTTTGCTCACCCA	717	-
TEM-R	TACGATACGGGAGGGCTTAC	-	Lal *et al*., 2007

Overnight cultures of donor and recipient cells were mixed at a ratio of 1:2 in 7 ml of Luria Bertani (LB) broth and incubated for 4 hours at 37 ^{o}C. Afterwards, lactose fermenting transconjugants were selected on MacConkey agar plates containing amoxicillin clavulanic acid and cefotaxime or ceftazidine (300µg/ml). Susceptibility tests and genotypic detection of ESBL genes by PCR were performed on the lactose fermenting transconjugants to confirm transfer as previously described. For controls, the donors and recipient were each grown separately in LB broth for 4 hours at 37 ^{o}C and the colony forming units of the recipient was counted.

Statistical/Data analysis

Differences in the prevalence of *P. aeruginosa* in the 2 locations sampled was determined using student unpaired T-test with p value of < 0.05 considered statistically significant.

RESULTS

Pseudomonas aeruginosa were isolated from 110 clinical samples from a total of 600 samples collected. The highest number were isolated from wound 48 (43.6%) followed by blood 44 (40.0%), then urine (18%) (Table 2), p = 0.743, T-test). Antimicrobial susceptibility tests reveal *Pseudomonas aeruginosa* had a high prevalence of resistance to amoxicillin - clavulanic acid, ceftazidime (an antipseudomonal drug) with cases of 100% resistance recorded in wound and blood samples. Resistance was also observed in *Pseudomonas aeruginosa* isolates tested with trimethoprim-sulfamethoxazole. The pattern of resistance observed in ciprofloxacin and gentamicin (antipseudomonal drugs) varied from one location and sample type to another and was equally high. Resistance of 70.6% and 45.5% were observed against gentamicin and ciprofloxacin, respectively in *Pseudomonas aeruginosa* isolated from wound and blood

samples. Some degree of *P. aeruginosa* susceptibility was however, observed when treated with nitrofurantoin (Table 3).

Results also showed that 77 (70.0%) of the *Pseudomonas aeruginosa* isolated were observed to be MDR strains. The frequency of occurrence of MDR strains was higher in wound (43.6%) and blood (40.0%) samples. Additionally, plasmid profile analysis revealed that all the 77 MDR isolates harboured plasmids of > I.5kp (Figure 1). The presence of multiple plasmids was observed. It is notable that similar plasmids sizes were observed in all MDR isolate harboring plasmids originating from wound and blood of GHA. Of 77 *Pseudomonas aeruginosa* isolates, 26 (33.8%) phenotypically ESBL- positive isolates were identified (Table 4). Among the 26 ESBL-positive isolates in phenotypic tests, the presence of 4(15.4%) *bla*TEM genes encoding ESBL was detected (Table 5). No *bla* SHV and *bla*CTX-*M* gene was detected.

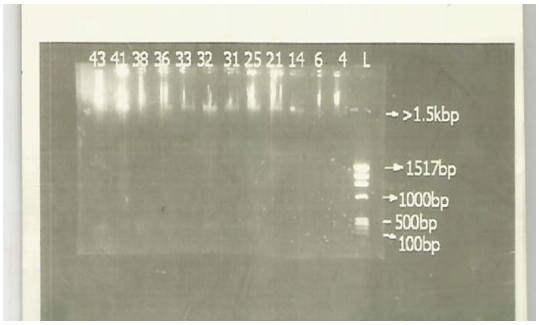

Figure1: plasmid profile of some *pseudomonas aeruginosa* isolates.
Lane L molecular laders, lanes 43, 41. 38, 36, 33, 32, 31, 25, 21,14, 6, 4 are *pseudomonas aeruginosa* isolates bearing plasmids of > I.5kp

To investigate the transferability of the plasmids, the 4 MDR *Pseudomonas aeruginosa* ESBL-positive genotypes were selected and conjugated with *E. coli* K-12. All *Pseudomonas aeruginosa* selected transferred their ESBL gene and resistant plasmids to *E. coli* K -12 recipient (Table 5).

Table 2: Distribution of clinical isolates of *Pseudomonas aeruginosa* obtained from Warri and Agbor General Hospitals, Delta State

	LOCATION			
SAMPLE TYPE	**WARRI n (%)**	**AGBOR n (%)**	**TOTAL (%)**	**p-VALUE**
Urine	14 (28.0)	4 (3.6)	18 (16.4)	0.742428
Wound	14(28.0)	34 (56.7)	48 (43.6)	
Blood	22 (44.0)	22 (36.7)	44 (40.0)	
Total	50 (45.5)	60 (54.5)	110 (18.3)	

DISCUSSION

In recent times, emergence of multidrug resistance (MDR) has jeopardized the use of many antibiotics and this is of huge concern. *Pseudomonas aeruginosa* with particular propensity for antibiotic resistance has been implicated in high mortality rates in many studies (Hirsch and Tam, 2010; Lambert *et al*., 2011). Equally reported is acquired resistance by the production of plasmid mediated ESBL enzymes (Manchanda and Singh, 2003). In this study, most of the isolates of *Pseudomonas aeruginosa* were obtained from wound and Blood (Table 2). Higher prevalence of MDR was also obtained from wound and blood. Various articles have associated *Pseudomonas aeruginosa with* increased mortality in blood stream infection (Peña *et al.,* 2012; Pachori *et al*., 2019; Recio *et al.,* 2020) and wound infection (Nasirmoghadas *et al.,* 2018).

Table 3: Susceptibility pattern of clinical isolates of *P. aeruginosa* obtained from Warri and Agbor General Hospitals, Delta State

LOCATION	SAMPLE	ANTIMICROBIAL AGENTS									
		A	CX	CZ	CR	CF	G	O	C	N	S
GHW	Urine	14(100.0)	6(42.9)	8(57.1)	8(57.1)	6(42.9)	6(42.9)	3(21.4)	3(21.4)	1(7.1)	8(57.1)
	Blood	12(54.6)	19(86.4)	19(86.4)	19(86.4)	12(54.6)	4(18.2)	4(18.2	2(9.1)	1(4.6)	12(54.6)
	Wound	14(100.0)	12(85.7)	14(100.0)	6(42.9)	8(57.1)	8(57.1)	2(14.3)	4(28.6)	4(28.6)	8(57.1)
GHA	Urine	4(100.0)	3(75.0)	3(75.0)	3(75.0)	2(50.0)	2(50.0)	0(0.0)	1(25.0)	0(0.0)	2(50.0)
	Blood	20(90.9)	20(90.9)	20(90.9)	20(90.9)	17(77.3)	12(54.6)	7(31.8)	10(45.5)	7(31.8)	9(40.9)
	Wound	29(85.3)	27(79.4)	34(100.0)	24(70.6)	12(35.3)	24(70.6)	10(29.4)	7(20.6)	8(23.5)	13(38.2)

A = amoxicillin-clavulanic acid; CX = cefixime; CZ = ceftazidime; CR = cefuroxime; CF = cefotaxime; G = gentamicin; O = ofloxacin; C = ciprofloxacin; S = trimethoprim-sulfamethoxazole; N = nitrofurantoin GHW = General hospital Warri; GHA = General hospital Agbor

Table 4: Prevalence of MDR and ESBL phenotype of *Pseudomonas aeruginosa*

HOSPITAL LOCATION	SAMPLE TYPE	PREVALENCE OF MDR SAMPLES n (%)	ESBL PHENOTYPE
Warri	Urine	9(11.7)	1(3.8)
	Wound	12(15.6)	10(38.5)
	Blood	10(12.9)	3(11.5)
Agbor	Urine	2(2.6)	0(0.0)
	Wound	25(32.5)	8(30.8)
	Blood	19(24.7)	4(15.4)
Total		77(70.0)	26(33.8%)

Table 5: Transfer of ESBL genotype in some selected *Pseudomonas aeruginosa* donor strains

DONOR STRAIN	RESISTANCE PATTERN	ESBL GENOTYPE
P.a 01	A CX CZ CF CR N G C O S	^{bla}TEM
P.a 02	A CX CZ CF CR G C O S	^{bla}TEM
P.a 03	A CX CZ CF CR G C O S	^{bla}TEM
P.a 04	A CX CZ CF CR G C O S	^{bla}TEM

P. a; *Pseudomonas aeruginosa*

The prevalence of multi-drug resistance *Pseudomonas aeruginosa* has increased over the last few decades and varies from one geographical area to another. Multi-drug resistantt *Pseudomonas aeruginosa* prevalence of 15 to 30% has been reported in most developed regions (Walkty *et al*., 2017; Sader *et al*., 2018) while lower prevalence was reported in Morocco (0%), Saudi Arabia (7.3%) and Qatar (8.1%) and high-level resistance was reported in Bahrain (86%), Egypt (75.6%), Lebanon (64.5%), Jordan (52.5%) and Palestine (47.6%) (Al-Orphaly, 2021). The low prevalence reported for instance in Saudi Arabia and Qatar was attributed to their well-structured health care systems as well as antibiotic-prescribing culture.

In contrast, this study reported 70.0% MDR *Pseudomonas aeruginosa*, this reflects the high usage of antibiotics coupled with the low rate of sensitivity testing to inform choice of antibiotic therapy. Antimicrobial stewardship programs are limited in low- and middle-income countries (Nathwani *et al*., 2019) and therefore, effective antimicrobial stewardship programs are crucial to the fight against spread of MDR, including *P. aeruginosa* (Tacconelli, 2018). The choice of antibiotics in our government owned hospitals is essentially based on treatment guidelines.

Resistance to antipseudomonal drugs such as ceftazidime actually reached an alarming level in this study. High prevalence of ceftazidime resistance in this study, ranged between 100 and 90.9% among wound and blood *Pseudomonas aeruginosa* isolates, respectively (Table 3). A similar study by Hussain *et al*. (2017) in Pakistan, ceftazidime resistance was 100%. Pakistan like Nigeria indulge in high frequency of drug use, antibiotics are easily available making resistance to antibiotics increasing rapidly (Solé *et al.,* 2015). High resistance to other 3rd generation cephalosporins was observed in this study and is attributable to ESBL production. *Pseudomonas aeruginosa* isolates were also resistant to the other two antipseudomonal agents (ciprofloxacin and gentamicin) determined in this study.

These drugs are used routinely as therapeutic and prophylactic treatment.

Twenty-six (33.8%) (Table 4) MDR *Pseudomonas aeruginosa* isolates produced ESBL by phenotypic method in this study. Comparable percentage prevalence of 37.2 was reported by Rani *et al.* (2016). Slightly higher and lower figures of 42.3% and 22.2% have been reported (Goel *et al*., 2013; Senthamarai *et al*., 2013). Genotypic detection of *Pseudomonas aeruginosa* isolates encoding ESBL genes from TEM family was observed in 15.4% in this study. The low prevalence may be because the ESBL enzymes found in *Pseudomonas aeruginosa* strains are primarily β-lactamases from the OXA, PER, GES (Potron *et al.,* 2015; Garza-Ramos *et al.,* 2015), VEB (Vatcheva-Dobrevska *et al.,* 2013; Potron *et al.,* 2015), BEL (Poirel *et al.,* 2005; Glupczynski *et al*.,2010; Potron *et al.,* 2015), and PME (Tian *et al*., 2011) ESBL family.

To ascertain the possible role of plasmid in the dissemination of beta lactamases resistance genes, the 4 genotypic ESBL positive *Pseudomonas aeruginosa* isolates were conjugated with *E. coli* K-12 recipient cell. The donor cells transferred their resistance genes to the recipient via conjugation and this is of grave implication as it exacerbates the spread of antibiotic resistance in the community thus threatening human health and increasing disease burden in the community. Of note is resistance to ceftazidime, ciprofloxacin and gentamicin observed in all the ESBL positive genes. This is due to the coexistence of genes encoding drug resistance to other antibiotics on the plasmids which encode ESBL (Nathisuwan *et al.,* 2001).

CONCLUSION

MDR *Pseudomonas aeruginosa* isolates were most common in blood and wound samples and were found to be resistant to the antipseudomonal drugs tested particularly ceftazidime. Although low prevalence of genotypic ESBL genes was observed, a high rate of transfer of plasmids harboring these genes was detected in this study.

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