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Analysis of Class 1 Integrons and Antibiotic Resistance Genes in *Pseudomonas aeruginosa* Strains from Benin City, Nigeria

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ABSTRACT: The presence of integrons and antibiotic resistance genes in the genome of *Pseudomonas aeruginosa* pose a serious problem in the treatment and control of infections caused by this pathogen in hospitals. This study was carried to analyse the presence of class 1 integrons and some antibiotic resistance genes on selected clinical and environmental strains of *Pseudomonas aeruginosa*. A total of 120 strains were employed for this study. The strains were confirmed using molecular method and species-specific primers targeting the 16S ribosomal ribonucleic acid (rRNA). Polymerase chain reaction (PCR) was used to detect the presence of class 1 integrons and resistance genes using appropriate primers and conditions. The strains were analysed for the presence of the following antibiotic resistance genes - *aadA*, bla_{PSE}, $bla_{AMP}C$, bla_{IMP} and *tetC* encoding aminoglycosides, beta-lactamases, metallo-beta-lactamases (MBL) and tetracylines resistance respectively. On screening the isolates for the presence of class 1 integrons, 50/60 (83.3 %) clinical isolates and 46/60 (76.7 %) environmental isolates showed positive results ($P > 0.05$). In both clinical and environmental isolates, the highest occurring resistance genes were $bla_{AMP}C$ and *tetC* (encoding beta-lactamases and tetracylines respectively), while the least was observed in bla_{IMP} (encoding metallo-beta-lactamases). In comparison, there was high significance difference (at $P<0.01$ significance level) in the resistance gene bla_{PSE} between the clinical and environmental strains. The high prevalence of these resistance genes is a great threat in the treatment of *Pseudomonas* infections.

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Pseudomonas aeruginosa is one of the most clinically significant bacteria. It is highly implicated in nosocomial infections in immuno-compromised patients. It has a natural resistance to β-lactams, including broad-spectrum cephalosporins, quinolones, chloramphenicol and tetracyclines, mainly due to their cell wall having low permeability. In addition to this mechanism, they possess inducible cephalosporinase, active efflux and poor affinity for the target (DNA gyrase) (Brown and Izundu, 2004). However, acquired extended spectrum β-lactamases (ESBL) and metallo-β-lactamases (MBL) mediated resistance is important emerging resistance mechanisms in *P. aeruginosa* (Lambert, 2002). These mechanisms thwart the use the treatment of infections using currently available drugs and anti-pseudomonal antibiotics.

The rapid dissemination of antibiotic resistance genes among bacterial isolates is an increasing problem in infectious disease. This organism has a high propensity to develop, acquire or transfer antimicrobial resistance genes (Gales *et al.,* 2001). This phenomenon is associated with increased rates of morbidity, mortality and high cost of treatment (Kohler *et al.,* 2001). Many resistance genes are present as gene cassettes within integrons, which may themselves be located on transmissible plasmid and transposon (Recchia and Hall, 1995).

Class I integrons (int-1) contribute to the spread of antimicrobial resistance genes and have been found in *Enterobacteriaceae* and other Gram-negative bacteria (Fluit and Schmitz, 1999), but they have also been found in a few Gram-positive bacteria. This study was carried out to determine the prevalence and distribution of class 1 integrons and relevant antibiotic resistance genes among the different *Pseudomonas aeruginosa* strains from clinical and environmental settings.

MATERIALS AND METHODS

Bacterial Isolates: One hundred and twenty (120) isolates of *P. aeruginosa* from diverse clinical and environmental sources were employed in this study. The clinical isolates (n = 60) were obtained from government-owned and private-owned hospitals in Benin City, Edo State, Nigeria. The isolates were obtained from patient's sputum, wounds, urine, blood,

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infected ears and eyes. The environmental isolates (n = 60) were from hospital environment, abattoirs and dump sites around Benin City. Standard microbiological methods were used to identify the isolates, as previously described by Isichei-Ukah and Enabulele (2018).

Isolation of Genomic DNA: Genomic DNA extraction was carried out by the method used by Odumosu *et al*. (2013). Briefly, the *P. aeruginosa* isolates were inoculated into 2 ml of tryptic soy broth and incubated overnight at 37 °C. The bacterial cells were harvested by centrifugation at 8,000 × g for 5 min and the supernatant was completely removed using sterile Pasteur pipette. The pellet was re-suspended in 500 µl of Tris EDTA (TE) buffer. The cells were lysed by boiling for 10 min in a water bath, cooled on ice, and centrifuged at 14,000 × g for 5 min to remove any cell debris before it was stored at −20°C. Aliquots of 2 µl of the template DNA were used for polymerase chain reaction (PCR).

Molecular Identification of Pseudomonas aeruginosa: Polymerase chain reaction (PCR) was used to confirm the identities of *Pseudomonas aeruginosa* using species-specific primers set. The primers used were: pa722F (5′-GGCGTGGGTGTG GAAGTC-3′) and pa899R (5′-TGGTGGCGATCTTGAACTTCTT-3′) amplicon size of 199 bp, which detects 16S rRNA for *P. aeruginosa* and PCR conditions (Lutz and Lee, 2011). *Pseudomonas aeruginosa* reference strain (ATCC 27853) was used as positive control.

Polymerase Chain Reaction for Detection of Class 1 Integrons: Polymerase chain reaction (PCR) was used to detect the presence of class 1 integrons in the *Pseudomonas aeruginosa* strains using the specific primer pairs: *int*1-F (5'-AAAACCGCCACTGCGCCGTTA3') and *int*1-R (3'GAAGACGGCTGCACTGAACG5'). The PCR conditions were: initial denaturation at 94º C for 12 min, 1 min of denaturation at 94ºC, 1 min of annealing at 55º C and 5 min of extension at 72º C for a total of 35 cycles (Fonseca *et al*. (2005). All reactions were set in 25 µl volume of reaction buffer containing 0.05 unit/µl *Taq* polymerase as directed by the manufacturer (Fermentas Life Sciences, USA). Electrophoresis of amplicons were performed with 2 % agarose gel containing 0.5 mg/L Ethidium Bromide (EtBr) (Merck, SA) for 1 h at 100 V in 0.5 × TAE buffer (40 mM Tris-HCl, 20 mM Na-acetate, 1 mM EDTA, pH 8.5) and visualized under an ultra-violet transilluminator.

Polymerase Chain Reaction for Detection of Antibiotic Resistant Genes: Polymerase chain reaction (PCR) was used to detect the presence of antibiotic resistance genes on the isolates. The resistances genes detected were *aadA* (encoding aminoglycosides), bla_{PSE} (encoding beta-lactamase), $bla_{AMP}C$ (encoding beta-lactamase), bla_{IMP} (encoding methallo-beta-lactamase) and *tet*C (encoding tetracyclines). The set of primers and PCR conditions used were earlier described by researchers: *aadA* (Fonseca *et al., 2005*), bla_{PSE} (Bert *et al., 2002*), $bla_{AMP}C$ (Yang *et al., 2008*), bla_{IMP} (Fonseca *et al., 2005*) and *tet*C (Agerso and Sandvang, 2005). The electrophoresis of the PCR reaction was carried out using 2% (w/v) agarose gel in 0.5 × TBE buffer applying a voltage of 100 V for 45 min and stained with ethidium bromide. Molecular weight marker of 100 bp was used as gene DNA Ladder. After electrophoresis, gel image was performed using imaging system UV transilluminator.

Statistical Analysis: All data were tabulated and then processed using *SPSS*, version 21.0. The distribution of resistance genes between clinical and environmental isolates was compared using the chi-square test. A *P*-value < 0.05 was considered statistically significant (Ogbeibu, 2005).

RESULTS AND DISCUSSION

Prevalence of Class 1 Integrons: By agarose gel electrophoresis PCR assay, clinical and environmental isolates were screened for the presence of class 1 integrons using *int*1 primer. Of the 60 clinical isolates tested, 50 (83.3%) were positive for class 1 integrons. For the environmental isolates (n=60), 46 (76.7%) isolates were positive (Table 1). There was no significant difference (P>0.05) in prevalence of class 1 integrons for clinical and environmental isolates.

Table 1: Prevalence of Class 1 Integrons in Clinical and Environmental Isolates of *Pseudomonas aeruginosa*

Integrase gene	Source	Prevalence n = 60	% Occurrence
***int*1**	Clinical	50	83.3
***int*1**	Environmental	46	76.7

$P > 0.05$

Class 1 integrons play an important role in transferring antibiotics resistance. Its prevalence is alarming for infections caused by this bacterium. The present study investigating the existence of class 1 integrons revealed 83.3% and 76.7% for clinical and environmental isolates respectively (Table 1). This is higher than previous reports of 57.4% from Southwest Nigeria (Odumosu *et al*., 2013). Other researchers had previously reported incidence rates of 41.5% from Brazil (Fonseca *et al*., 2005), 45.8% from China (Xu *et al*., 2009), and 56.3% from Iran (Yousefi *et al*., 2010).

In Africa, class 1 integrons had previously been reported in clinical isolates of *P. aeruginosa*. Labuschagne *et al*. (2008) reported bla_{GES-5} and bla_{GES-5}-like genes as part of the variable region of class 1 integrons, occurring in three clinical *P. aeruginosa* isolates from South Africa. Another study reported class 1 integron containing bla_{VIM-2}, *aacA7* and *aacA4*, as well as *aadB* and *arr6*, a novel rifampin resistance gene among 35 clonally related *P. aeruginosa* isolated from a hospital in Tunisia (Hammami *et al*., 2010).

Although there was no significant difference between clinical and environmental isolates (P>0.05) in this study, the clinical isolates had a higher prevalence of class 1 integrons. This suggests that class 1 integrons are strongly associated with multiple drug resistance and are frequently detected among clinical isolates of *P. aeruginosa* (Martinez *et al*., 2012). The prevalence of class 1 integrons in the isolates of *P. aeruginosa* from this region is of great concern because these genetic elements are highly stable among resistant pathogens, and also capable of easy spread and capture of other multidrug resistance gene cassettes which may lead to increase in resistance to broad spectrum antibiotics (Tenover, 2006).

Prevalence of Resistance Genes: The percentage occurrence of the resistance genes in the clinical isolates were shown (Table 2). Among the five resistance genes, bla_{AMP} had the highest prevalence of 95.0%; and the least was in bla_{IMP} (3.3%). There was very high significant difference in prevalence among resistance genes in the clinical isolates (P<0.001).

For the environmental isolates, bla_{AMP} also had the highest prevalence of 98.3%. Resistance gene, $bla_{IMP,}$ had the least prevalence of 5.0% (Table 3). There was very high significant difference in prevalence among resistance genes in the environmental isolates (P<0.001).

The comparison of each of the antibiotic resistance genes between the clinical and environmental isolates are shown in Figure 1. There was no significant difference in prevalence between the clinical and environmental isolates (P>0.05) for all resistance genes, except for bla_{PSE} where there was high significant difference (at P<0.01).

Table 2: Prevalence of Antibiotic Resistance Genes in Clinical Isolates of *Pseudomonas aeruginosa*.

Resistance Genes	Antibiotic Class	Occurrence (%) n = 60
aadA	Aminoglycosides	12 (20.0)
$\boldsymbol{bla_{PSE}}$	Beta-lactams	28 (46.7)
$\boldsymbol{bla_{AMP}C}$	Beta-lactams	57 (95.0)
$\boldsymbol{bla_{IMP}}$	Carbapenems	2 (3.3)
tetC	Tetracycline	48 (80.0)

Table 3: Prevalence of Antibiotic Resistance Genes in Environmental Isolates of *Pseudomonas aeruginosa*

Resistance Genes	Antibiotic Class	Occurrence (%) n = 60
aadA	Aminoglycosides	10 (16.7)
$\boldsymbol{bla_{PSE}}$	Beta-lactams	13 (21.7)
$\boldsymbol{bla_{AMP}C}$	Beta-lactams	59 (98.3)
$\boldsymbol{bla_{IMP}}$	Carbapenems	3 (5.0)
tetC	Tetracycline	46 (76.7)

From the results (Tables 2 and 3), the prevalence of the target resistance genes were observed. This finding correlated with the works of Igbinosa and Obuekwe (2014); who detected the presence of some resistance genes to be bla_{IMP} (14.7%), $bla_{AMP}C$ (14.7%), and *tetC* (5.8%) in *P. aeruginosa* isolates from abattoir environment around this region (Benin City, Nigeria). The presence of *aadA* genes (encoding aminoglycosides resistance) was 20% and 16.7% for clinical and environmental isolates respectively. There was no significant difference between clinical and environmental isolates (P>0.05).

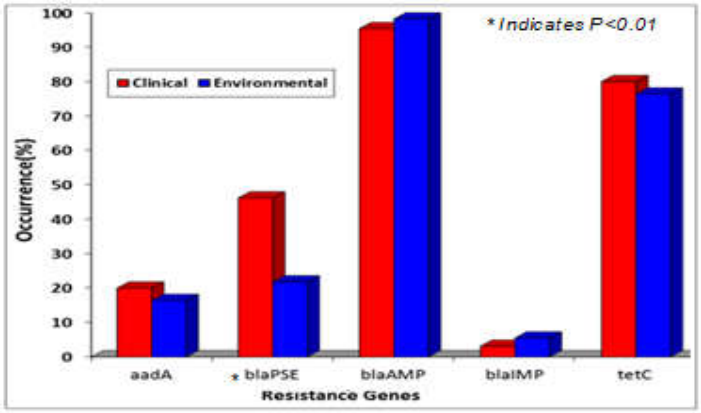

Fig 1: Prevalence of Antibiotic Resistance Genes in Clinical and Environmental Isolates of *Pseudomonas aeruginosa*

From this study, the prevalence of $bla_{AMP}C$ (encoding beta-lactam resistance gene) was 95% and 98.3% for clinical and environmental isolates respectively. There was no significant difference between clinical and

environmental isolates (P>0.05). But for bla_{PSE}, also encoding beta-lactam resistance, had prevalence of 46.7% and 21.7% for clinical and environmental isolates respectively. There was high significant difference between clinical and environmental isolates (P<0.01). The clinical isolates had a higher prevalence. This correlates with the works of Bert *et al*. (2002) who detected 62.5% of bla_{PSE} resistance genes in clinical isolates in France. The presence of bla_{IMP} genes (encoding metallo-*β*-lactam or carbapenem resistance genes) was 3.3% and 5% for clinical and environmental isolates respectively. There was no significant difference between clinical and environmental isolates (P>0.05). Among the other resistance genes tested, bla_{IMP} genes had the lowest prevalence. The existence of metallo-*β*-lactamases and extended-spectrum *β*-lactamase-producing strains exhibiting resistance to most *β*-lactams antimicrobial agents greatly complicate the clinical management of patients infected with such multi-drug-resistant strains (Moreira *et al.* 2002; Pagani *et al.* 2002). The prevalence of *tetC* gene (encoding tetracycline resistance) in this study was 80% and 76.7% for clinical and environmental isolates respectively. There was no significant difference between clinical and environmental isolates (P>0.05). This is in contrast with the work of Igbinosa and Obuekwe (2014), which had a prevalence of 5.8% in environmental isolates in this region. The presence of this resistance genes in the isolates could be attributed to the fact that this antibiotic is used indiscriminately in our environment and could also be as a result of the isolates possession of an intrinsic and acquired resistance mechanism caused mainly by an active efflux system, which efficiently expels the compound from the cell (Kohler *et al.,* 2001).

*Conclusion***:** The high prevalence of class 1 integrons and resistance genes is really alarming and this can be great threat in the control and treatment of pseudomonal infections. However, this can result in increased length of hospitalization and mortality. Therefore, practical steps should be taken in the proper use of antibiotics in both clinical and environmental settings.

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