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Evaluation of Antibiotic Resistant Gene in Abattoir Environment

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***KEYWORDS*:** Abattoir, Class 1 integron, *Pseudomonas aeruginosa*, Multi-resistance, Gene cassette

ABSTRACT: *Pseudomonas aeruginosa* is a ubiquitous gram-negative pathogen with susceptibility to cause opportunistic infections in humans. Among the total of 75 isolated presumptive *Pseudomonas aeruginosa*, by cultural and morphology characteristic, 55 were identified as *Pseudomonas aeruginosa* by the specie-specific primer employing PCR assay which was more sensitive for confirmation of the isolates. Fifty-five *Pseudomonas aeruginosa* isolates were screened using PCR for the presence of integrons and associated resistance gene cassette. Thirty-four isolates harbored class 1 integrons (61.8%), of which 27 isolates (79.4%) carried gene cassettes. PCR was performed targeting the presence of these genes:- bla_{OXA} 11(32.4%); bla_{IMP} 5(14.7%); $bla_{AMP}C$ 5(14.7%); bla_{TEM} 4(11.8%); and *TetC* 2(5.8%). Some of these genes were only recently described from clinical isolates, demonstrating genetic exchange between clinical and environmental *Pseudomonas aeruginosa* stains. Multi-resistance was observed in the isolates, revealing strong correlation between integron presence and multi-resistance. These results demonstrate that abattoir milieu is potential reservoirs of various antibiotics resistance genes, thus constituting a serious health risk to the communities dependent on the receiving water bodies. © JASEM

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Introduction: Pseudomonas aeruginosa is a leading cause of hospital-acquired infections, giving rise to a wide range of opportunistic infections. Its high intrinsic resistance to antibiotics and ability to develop multidrug resistance pose serious therapeutic problems (Kohler *et al.,* 1999). *Pseudomonas aeruginosa* is highly ubiquitous in water systems, and has intrinsic antimicrobial resistance due to low outer membrane permeability, as well as an extensive efflux pump system (Aeschlimann 2003; Lister *et al.,* 2009). *P. aeruginosa* demonstrates resistance to multiple antibiotics, thereby rendering common antibiotic therapy ineffective (Bodey *et al.,* 2008). The presence of multidrug-resistant *P. aeruginosa* in an aquatic milieu may be important for immune-suppressed or other at-risk individuals, for whom treatment difficulties have greater implications (Obritsch *et al.,* 2004). *P. aeruginosa* frequently acquires additional resistance mechanisms (plasmids) and routinely develops multidrug resistance throughout the course of a treatment regimen (Lister *et al.,* 2009).

The integrase (*Int1*) is the signature of an integron. To date, three classes of integrons (class 1, 2, and 3) have been described to be associated with resistance gene cassettes (Strokes and Hall, 1989). Class 1 is recognized as the most widespread among environmental isolates (Recchia and Hall, 1995; Collis *et al.,* 1998). Various resistance associated genes are harbored in class 1 integron found in *P. aeruginosa*, including those encoding extended-spectrum–β-lactamases (ESBLs) and metallo-β-lactamases that hydrolyse third and fourth generations of cephalosporins and carbapenems respectively (Weldhagen *et al.,* 2004).

Naturally, this pathogen is endowed with weak pathogenic potentials. However, its profound ability to survive on inert materials, its minimal nutritional requirement, tolerance to a variety of physical conditions and its relative resistance to several unrelated antimicrobial agents and antiseptics, contributes enormously to its ecological success and its role as an effective opportunistic pathogen (Gales *et al.,* 2001). The organism is pathogenic when introduced into area devoid of normal defence (Jawetz *et al.,* 1991) and its infections are both invasive and toxigenic (Todar, 2005). It has been widely reported that the susceptibility of this pathogens to antibiotics varies with time and geographical location. *P. aeruginosa* accounts for significant proportion of nosocomial infections and the tendency of nosocomial pathogen to develop or acquire new antibiotics resistance traits poses a great problem in their treatment and control.

Igbinosa *et al.* (2012) reported that multidrug resistance in *P. aeruginosa* population is a pervasive and growing environmental problem, which is recognized as a threat to public health. Consequently, there is a need to conduct area-specific monitoring studies to profile different pathogens responsible for specific infections and their resistance patterns, so as to generate data that would help clinicians to choose the correct empirical treatment. This paper reports the

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occurrence of some antibiotic resistant genes in *Pseudomonas aeruginosa* recovered from abattoir environments in Benin City, Nigeria.

MATERIALS AND METHODS

Bacterial Strain: A total of Seventy-five presumptive *Pseudomonas aeruginosa* were isolated from abattoir environment in our previous work (Igbinosa *et al.*, 2012). The isolate identification was determined by phenotypic characteristics and standard biochemical reaction using API 20 NE system (bioMerieux, Marcy l'Etoile, France).

Antimicrobial Susceptibility Testing: The antimicrobial susceptibility was carried out as described by Igbinosa et al. (2012). Agar dilution susceptibility testing was employed. Amikacin, aztreonam, cefepime, ciprofloxacin, piperacillin-tazobactam, ceftazidime, imipenem and meropenem were used to prepare antibiotic stock solutions as described by CLSI (2006). A 1:10 dilution was made of each antibiotic stock solution to be tested to obtain a final concentration of 2,560 µg/ml. An agar dilution series (0.125-512 µg/ml) was set up according to CLSI (2006) procedure. Colonies of overnight culture on Mueller-Hinton agar medium were used to prepare and adjust inoculums as described by CLSI (2006). A 200 µl of each microbial suspension was placed into the wells of an inoculum. A growth control agar plate without antibiotics was inoculated first; thereafter all plates were inoculated starting with the lowest concentration. The inoculated spots were left to dry after which the inoculated agar plates were incubated at 37^{o}C for 18 to 24 h.

Isolation of Genomic DNA: Genomic DNA was extracted following a modified scheme of Igbinosa et al. (2012). Single colonies of *P. aeruginosa* strains grown overnight at 37^{o}C on nutrient plates were picked, suspended in 100 µl of sterile double distilled water and the cells were lysed using Heat Block for 15 min at 100^{o}C. The cell debris was removed by centrifugation at 11,000 *g* for 2 min using a MiniSpin micro centrifuge and the supernatant used directly as template DNA or stored at -20^{o}C until ready for use.

Specie-specific Identification: PCRs were performed in 22.5 µl volume of reaction buffer containing 0.05 unit/ml Taq polymerase as recommended by the manufacturer (Fermentas Life Sciences) and 2.5 µl of DNA template. Sterile double distilled water was included in each PCR assay as a negative control and positive controls contained DNA templates of *P. aeruginosa* ATCC 27853. All PCR was conducted using a MultiGene Thermal Cycler (Labnet International Inc., Edison, NJ, USA), at the following conditions: 95ºC for 1 min; 40 cycles of denaturation at 95ºC for 15 s, annealing at 58ºC for 20 s; final extension at 68ºC for 40 s and holding temperature of 4^{o}C. The primers used were: pa722F (5'-GGC GTG GGT GTG GAA GTC-3') and pa899R (5'-TGG TGG CGA TCT TGA ACT TCTT-3') amplicon size of 199 bp (Lutz and Lee, 2011). Electrophoresis of amplicons was performed with 1% agarose gel (Hispanagar, Spain) containing ethidium bromide (EtBr) 0.5 mg/L (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 UV transilluminator (BioDoc-It System, UVP Upland, CA 91786, USA).

Detection of Antibiotics Resistance Genes: Polymerase chain reaction (PCR) was used to detect antibiotic resistant elements in the *P. aeruginosa* using the specific primers. PCR conditions for detection of the class 1 integrons was determined as described elsewhere (Fonseca *et al.*, 2005), while bla_{IMP}, bla_{OXA}, bla_{TEM}, *Tet(C)*, and $bla_{AMP}C$ genes were based on protocols listed in Table 1. PCRs were performed in 45 µl volume of reaction buffer containing 0.05 unit/ml Taq polymerase as recommended by the manufacturer (Fermentas Life Sciences) and 5 µl of DNA template. Sterile Milli-Q PCR grade water (Merck, SA) was included in each PCR assay as a negative control. Cycling conditions (MultiGene Thermal Cycler) were as follows for bla_{IMP}, and bla_{OXA}, genes: - initial denaturation at 94^{o}C for 5 min followed by 30 cycles of denaturation at 96^{o}C for 30 s, annealing at 55^{o}C for 30s and elongation at 72^{o}C for 1 min with a final extension at 72°C for 5 min. bla_{TEM} gene (3 min at 93°C, 40 cycles of 1 min at 93°C, 1 min at 55°C and 1 min at 72°C and finally 7 min at 72°C); $bla_{AMP}C$ gene (94°C for 5 min, 30 cycles of 25 s of denaturation at 94°C, 40 s of annealing at 53°C and 50 s of extension at 72°C and a final cycle at 7 min at 72°C); *TetC* gene (3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 65°C and 1 min at 72°C followed by 10 min at 72°C). Ten microlitres (10 µl) of reaction mix containing PCR products was analysed by electrophoresis in 1% (w/v) agarose.

RESULTS AND DISCUSSION

Integron Detection and Resistance Genes Characterization: A total of 75 presumptive *P. aeruginosa*, by cultural and morphology characteristic were isolated, 55 were identified as *P. aeruginosa* by the specie-specific primer employing PCR assay which was more sensitive in the confirmation of the isolates. All 55 *P. aeruginosa* isolates tested for susceptibility to antibiotics were screened for the presence of class 1 integron. Thirty-four isolates harbored class 1 integrase (61.8%), of which 27 isolates (79.4%) carried resistance genes associated with class 1 integron. In order to establish the prevalence of these genes in association with class 1 integron, PCR was performed targeting the presence of these genes: - bla_{OXA} 11(32.4%); bla_{IMP}

5(14.7%); $bla_{AMP}C$ 5(14.7%); bla_{TEM} 4(11.8%); and *TetC* 2(5.8%) (Table 2).

The rapid dissemination of antibiotic resistance genes among bacterial isolates is an increasing problem in infectious disease. *Pseudomonas aeruginosa* is a leading cause of nosocomial infections with 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). Previous studies have shown that a conserved DNA sequence, integron may be carried on these episomal genetic structure (Stokes and Hall, 1989; Rowe-Magnus and Mazel, 1999). Integrons possess two conserved segments separated by a variable region that includes integrated cassettes, which often include antibiotic resistance genes (Recchia and Hall, 1995). Many resistance genes are present as gene cassettes within integrons, which may themselves be located on transmissible plasmid and transposon (Recchia and Hall, 1995). Our study revealed that integrons were widely distributed among environmental isolates of *P. aeruginosa* from abattoir locale in Benin City, Nigeria with integrase gene amplicons obtained from 61.8% (34/55) (Table 2). This is comparable with previous reported frequencies of 41.5% in Brazil (Fonseca *et al.,* 2005), 60% in the United Kingdom (*A. baumannii*) (Turton *et al.,* 2005), 43% in Europe (gram-negative isolates) (Martinez-Freijo *et al.,* 1998), >50% in The Netherlands (*Enterobacteriaceae*) (Jones *et al.,* 1997), 59% in France (*Enterobacteriaceae*) (Sallen *et al.,* 1995), and 52% in Taiwan (*Escherichia coli*) (Chang *et al.,* 2000).

In this study we compared susceptibility data from integron positive *P. aeruginosa* isolates. It was observed that integrons were significantly associated with resistance to certain antibiotics, including aminoglycosides, quinolones and beta-lactam antibiotics. This is not surprising, since many antibiotics resistance gene cassette encoding resistance to a wide range of antibiotics have been reported previously (Sallen *et al.,* 1995; Rowe-Magnus and Mazel, 1999; Igbinosa *et al.,* 2012). Multiple antibiotics resistance defined as resistance to six or more antibiotics, correlated strongly with the presence of integrons (Table 2). It is well-known that multiple mechanisms are related to antibiotic resistance in *P. aeruginosa* (Livermore, 2002), and the integrons role in this species is an additional element in the dynamics of the resistance acquisition.

Multi-drug resistance in environmental isolates might be linked to the uncontrolled disposal of antibiotics and chemicals into the environment creating a selective pressure on these drugs. The use of antibiotics in hospital and the community at large serve as major selective pressure for antibiotics resistant bacteria (Moreira *et al.,* 2002).Nosocomial infections are increasing globally as a result of multi drug resistant pathogens. The existence of metallo-β-lactamases and extended-spectrum β-lactamase exhibiting resistance to most -β-lactams antimicrobial agents greatly complicate the clinical management of patients infected with such multi-drug-resistance strains (Moreira *et al.,* 2002; Pagani *et al.,* 2002). The presence of *tetC* gene (tetracycline) in the isolates could be attributed to the fact that this antibiotic is used indiscrimately 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). *P. aeruginosa* resistance to antibiotics is a serious problem in clinical and environmental locale in Africa. Similar studies conducted in South Africa, Cote d'Ivoire, Tunisia and Nigeria (Aka *et al.,* 1987; Rotimi *et al.,* 1994; Poirel *et al.,* 2001, 2002; Igbinosa *et al.,* 2012) documented the existence of mult-resistant strain of *P. aeruginosa* responsible for nosocomial infections. In Nigeria, carbapenems (imipenem), beta-lactam antibiotics plus beta-lactamase inhibitors (piperacillin-tazobactam), aminoglycosides (amikacin), and quinolones (levofloxacin) have often been used to cure *P. aeruginosa* infections (Igbinosa *et al.,* 2012). Our findings thus showed that integron gene element bearing drug resistance markers were moderately distributed in the *P. aeruginosa* strains isolated from our study site. It also revealed the frequency of occurrence of the gene cassettes, bla_{OXA}, bla_{IMP}, bla_{AMPC}, bla_{TEM}, and *TetC*. It is important to monitor the distribution of integron gene in emerging *P. aeruginosa* strains.

Conclusion: To the best of our knowledge, this is the first study that describes the detection of antibiotics resistance genes known to confer resistances to common classes of antibiotics in an abattoir environment in Benin City, Nigeria. These results demonstrate that abattoir effluents are potential reservoirs of various antibiotics resistance genes. Moreover, detection of resistance genes in *P. aeruginosa* strains obtained from the abattoir effluents suggests that these resistance determinants might be further disseminated in habitats downstream, thus constituting a serious health risk to the communities dependent on the receiving water bodies.

Table 1: Sequence of primers used for detection of antibiotics resistance genes

Primer	Nucleotide sequence (5' to 3')	Target gene	Amplicon size bp	Reference
bla_{AMP} C-F	GGTATGGCTGTGGGTGTTA	*Bla_{AMP}* C gene	882	Yang *et al.* (2008)
bla_{AMP} C-R	TCCGAAACGGTTAGTTGAG			
TetC -F	GGTTGAAGGCTCTCAAGGGC	*Tet* C gene	505	Agersø and Sandvang, (2005)
TetC -R	CCTCTTGCGGGATATCGTCC			
bla_{TEM}-F	AGGAAGAGTATGATTCAACA	*Bla_{TEM}* gene	535	Wang *et al.* (2006)
bla_{TEM}-R	CTCGTCGTTTGGTATGGC			
Imp-F	CTACCGCAGCAGAGTCTTGG	*Bla_{IMP}* gene	Variable	Fonseca *et al.* (2005)
Imp-R	AACCAGTTCTGCCTTACCAT			
Oxa-F	AAGAAACGCTACTCGCCTGC	*Bla_{OXA}* gene	478	Bert *et al.* (2002)
Oxa-R	CCACTCAACCCATCCTACCC			
Int 1-F	AAAACCGCCACTGCGCCGTTA	Class 1 integrase	Variable	Fonseca *et al.* (2005)
Int 1-R	GAAGACGGCTGCACTGAACG			

Table 2: Phenotypic and genotypic characterization of *Pseudomonas aeruginosa* strains and their antibiotics resistance genes

Isolates code (n = 55)	Resistance profile [a]	Strain(s) showing presence of gene encoding					
		Bla_{IMP}	*Bla_{OXA}*	*Bla_{TEM}*	*Tet(c)*	*$Bla_{AMP}C$*	Class 1 integrase
DPT14	Ami, Azt, Mem, Pip, Cef	-	+	-	-	+	+
DPT23	Azt, Pip, Imi, Cef	-	-	-	-	-	-
DPT18	Ami, Pipt, Azt, Mem	-	+	+	-	-	+
DPT40	Ami, Azt, Ceft, Pip	-	-	-	-	-	-
DPT25	Ami, Imi, Mem, Azt	-	-	-	+	-	+
DPT15	Azt, Imi, Cet, Pip	-	-	-	-	-	-
DPT8	Ami, Mem, Pipt, Imi, Pip	+	-	-	-	-	+
DPT11	Cef, Imi, Mem, Pipt	-	-	-	-	-	+
UPST30	Ami, Pipt, Azt, Mem	-	-	-	-	-	+
UPST45	Ceft, Azt, Pip, Ami, Cef	-	-	+	-	-	+
UPST5	Ceft, Imi, Azt, Pipt	-	+	-	-	-	+
DWST31	Ami, Ceft, Azt, Mem	-	-	-	+	-	+
DWST51	Ami, Azt, Mem, Pip	-	-	-	-	-	-
DWST2	Ami, Mem, Imi, Pipt	-	-	-	-	-	-
DWST30	Ami, Imi, Cef, Azt	-	-	-	-	-	+
DWST18	Imi, Azt, Ceft, Ami	-	-	-	-	-	+
DWST34	Imi, Azt, Ceft, Pip Cef	-	-	-	-	-	+
DWST6	Imi, Ami, Ceft, Azt	-	-	-	-	-	+
DWST9	Mem, Imi, Ami, Pip, Azt, Cef	+	+	-	-	+	+
DWST16	Mem, Imi, Pip, Ceft	-	-	-	-	-	-
DWST37	Mem, Imi, Azt, Cef	-	-	-	-	-	+
DWST42	Pip, Cef, Imi, Ami	-	-	-	-	-	-
DWST28	Azt, Imi, Pipt, Pip	-	-	-	-	-	+
UPST35	Ami, Pipt, Pip, Cef	-	-	-	-	-	-
UPST39	Azt, Imi, Ami, Cef, Ceft	+	+	-	-	+	+
UPST27	Imi, Ami, Azt, Mem	-	+	-	-	-	+
ABSU5	Mem, Azt, Cef, Ceft	-	-	-	-	-	-
ABSU9	Imi, Ami, Pip, Azt, Ceft	+	-	+	-	+	+
ABSU36	Ami, Azt, Cef, Ceft	-	-	-	-	-	-
ABSU48	Cef, Mem, Pip, Ami	-	-	-	-	-	-
ABSU23	Ceft, Imi, Pipt, Azt	-	+	-	-	-	+
ABWT33	Ami, Imi, Mem, Azt	-	-	-	-	-	-
ABWT8	Azt, Imi, Cef, Pip	-	-	-	-	-	+
ABWT40	Ami, Mem, Azt, Cef	-	-	-	-	-	-
ABWT45	Cef, Mem, Imi, Pip	-	-	-	-	-	-
DPT10	Azt, Mem, Cef, Ami, Pip	-	+	-	-	-	+
DPT5	Cef, Pip, Imi, Azt,	-	-	-	-	-	+
DPT15	Ami, Azt, Mem, Pipt	-	-	-	-	-	-
DPT45	Ami, Azt, Ceft, Pip	-	-	-	-	-	+
DPT2	Azt, Mem, Imi, Ami	-	-	-	-	-	-
UPSTa	Mem, Ami, Pipt, Azt	-	-	-	-	-	+
UPSTc	Azt, Ceft, Pip, Cef Ami,	-	-	-	-	-	+
UPSTx	Pipt, Ceft, Azt, Imi	-	-	-	-	-	+
UPSTy	Pipt, Ami, Cef, Pip	-	-	-	-	-	-
UPSTz	Ceft, Imi, Azt, Cef, Ami	-	+	-	-	-	+
DWSTc	Azt, Ami, Mem, Ceft	-	-	-	-	-	+
DWSTd	Ami, Pip, Azt, Mem	-	-	-	-	-	-
DWSTz	Ami, Imi, Azt, Ceft	-	-	-	-	-	-
DWSTy	Imi, Mem, Azt, Pip, Cef, Ami	+	+	+	-	+	+
DWSTq	Cef, Mem, Imi, Azt	-	-	-	-	-	+
ABWTs	Mem, Azt, Cef, Ceft	-	-	-	-	-	+
ABWTd	Imi, Ami, Pip, Azt, Ceft	-	+	-	-	-	+
ABSUx	Ami, Imi, Mem, Azt	-	-	-	-	-	-
ABSUz	Azt, Imi, Cef, Pip	-	-	-	-	-	+
ABWTy	Cef, Mem, Pip, Ami	-	-	-	-	-	-
% Positive		5(14.7%)	11(32.4%)	4(11.8%)	2(5.8%)	5(14.7%)	34(61.8%)

Legend: Ami-Amikacin; Azt-Aztreonam; Pip-Piperacillin; Pipt-Piperacillin-tazobactam; Cef-Cefepime; Ceft-Ceftazidime; Imi-Imipenem; Mem-Meropenem [a]Extracted from Igbinosa et al. (2012) The Scientific World Journal doi:10.1100/2012/308034

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