

Occurrence of Integron Genes in Multiple Drug Resistant Strains of *Pseudomonas aeruginosa* in Benin City, Nigeria

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Abstract: *Pseudomonas aeruginosa* is an opportunistic pathogen with the ability to cause severe surgical wound infections and remains a problem. This microorganism commonly shows resistance to several antibiotics. Integron a mobile genetic elements are playing important functions in the wide spread of *P. aeruginosa* antibiotic resistance. This study is aimed at investigating the occurrence of class 1, 2 and 3 integron genes (*int1*, *int2*, *int3*) among *P. aeruginosa* strains. For this purpose, a total of 284 wound swabs were collected using sterile swab sticks. Isolated *P. aeruginosa* were screened with 8 routinely used antibiotics by means of disk diffusion method. Polymerase chain reaction amplification was carried out on extracted DNAs from *P. aeruginosa* for the detection of integron and subsequent classification into *int1*, *int2* and *int3* genes using different set of specific primers. Out of the 99 isolates seen, 62 (66.7%) were *P. aeruginosa*. Most isolates that harbors integron genes showed notable resistance to antibiotics with highest resistance against Ceftazidime, Augmentin, Cefixime and Gentamicin (54.8%). PCR amplification showed that 16 (47.1%) *P. aeruginosa* strains harbors integron genes of which 13 (81.3%) isolates carried *int1* gene, 8 (50.0%) and 6 (37.5%) harbored *int2* and *int3* genes respectively. High antibiotic resistance amongst *P. aeruginosa* isolates were demonstrated in our study, *int1* gene was prevalent followed by *int2* then *int3* and integrons has been reported to play an important role in multiple drug resistance among bacteria isolates.

Keywords: Antimicrobial, wound swabs, infections, post operative, polymerase chain reaction.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic disease causing microorganism with the possibility to cause severe healthcare-related infections particularly in immune-compromised post surgical wound patients (Mesaros *et al.*, 2007; Strateva and Yordanov, 2009). Despite advances and improvement in aseptic techniques in surgical procedures, wound infections still constitute common occurrences (Yah *et al.*, 2009). Generally speaking, surgical wounds through repression of body defense mechanism offer acceptable site for microorganism multiplication. Therefore, *P. aeruginosa* infection in surgical site infection patients is common and is seen as one of the foremost serious life threatening conditions in surgical operation units (Church *et al.*, 2006). Furthermore, due to the resistance of these microorganisms to a wide range of antibiotics in recent years, treatment of infections caused by them has been worrisome and has resulted in

increased mortality rate (Fair and Tor, 2014). The difficulty in the treatment of *P. aeruginosa* infection is because of its acquired and intrinsic resistance to totally different antibiotics caused by many mechanisms as well as low outer membrane permeableness, over expression of efflux pump system and protein modifications (Breidenstein *et al.*, 2011). Distribution of antibiotic resistance genes by mobile genetic components is an ever-increasing concern worldwide which has resulted in increased number of multi-drug resistant (MDR) strains among *P. aeruginosa* isolates (Sun *et al.*, 2014).

Integrons are genetic components which are connected to transposons, plasmids and chromosomal deoxyribonucleic acids which are responsible for development of microorganism resistance among gram-negative bacteria pathogens (Fluit and Schmitz, 2004; Gillings, 2014).

Integrans are composed of three vital core elements: The *intI* gene which encodes an integrase (*IntI*), required for site-specific recombination; *attI*, the flanking recombination site that is recognized by integrase; and integrin related promoter (*Pc*), which is needed for transcription and expression of gene cassette inside the integron. Gene cassettes are genetic components that code antibiotic resistance, and incorporate a specific site recombination known by integrase which is referred to as *attC* (Domingues *et al.*, 2012). Based on the variations within the amino acid sequences of encoded integrases, integrons have been divided into 5 categories (Cambray *et al.*, 2010; Deng *et al.*, 2015). The class 1 integron with the best assortment of sequence cassettes, is the most clinically relevant and features among wide range of resistant strains (Deng *et al.*, 2015). Because of the significance of integrons in the wide spread of antibiotic resistance, this study is aimed at understanding the incidence of class 1, 2 and 3 integrons among *P. aeruginosa* strains isolated from post surgical site infection in Benin City, Nigeria.

MATERIALS AND METHODS

Sample collection

A total of 284 random swab sampling of patients with post operative surgical wound was collected from both inpatient and outpatient in Benin City, Nigeria with respective bio-data.

Ethical clearance

Approval was obtained from the University of Benin Teaching Hospital and Central Hospital, ethical committees and all patients gave their approval after being informed of the objectives of the study.

Bacteriological procedures/identification of isolates

Specimens were aseptically inoculated onto MacConkey, Blood and Nutrient agar and incubated aerobically at 37 °C for 24 hours and observed for colonial growth. Isolates were screened for the presence of *Pseudomonas aeruginosa*. All specimens were processed at Lahor research Laboratories, Benin City, Nigeria using

standard microbiological methods. All isolates were identified using conventional techniques (Cheesbrough, 2000).

Antibiotic susceptibility testing

The susceptibility of isolates to frequently used antibiotics were determined by the Kirby-Bauer disk diffusion method for *in vitro* antibiotic susceptibility as described by NCCL (2002), against the following antibiotics for Gram negative bacteria which include: Augmentin (AUG, 30µg), Ofloxacin (OFL 5µg), Cefixime (CXM 5µg), Gentamycin (GEN 30µg), Cefuroxime (CRX 30µg), Ceftazidime (CAZ 30µg), Ciprofloxacin (CPR 5µg), Nitrofurantion (NIT 300µg). The concentrations of antibiotics susceptibility and interpretation of zones of inhibition were in accordance to Performance Standards for antimicrobial disk susceptibility tests of Clinical and Laboratory Standards Institute (CLSI, 2011). Commercially available antibiotics were used for this study.

Bacteria genomic DNA extraction

Multiple drug resistant *Pseudomonas aeruginosa* strains were subcultured overnight in Luria-Bertani broth (Merck, Germany) and genomic DNA was extracted from typical colonies of *Pseudomonas aeruginosa* using Zymo research fungi/bacterial DNA MiniPrep DNA extraction kit (Irvine, CA, USA), according to manufacturer's instructions.

Detection of integron genes in *Pseudomonas aeruginosa* using polymerase chain reaction technique

Polymerase Chain Reaction (PCR) was used for the amplification of integron genes in *Pseudomonas aeruginosa*. Forward and reverse primers for integron class 1, 2 and 3 (*Int1*-F-GCCACTGCGCCGTTACCACC; *Int1*-R-GGCCGAGCAGATCCTGCACG, *Int2*-F-CACGGATATGCGACAAAAAGGT; *Int2*-R-GTAGCAAACGAGTGACGAAATG, *Int3*-F AGTGGGTGGCGAATGAGTG; *Int3*-R-TGTTCTTGTATCGGCAGGTG) (Sunde, 2005; Mohammadalipour *et al.*, 2017) were amplified separately in a ABI9700 thermal cycler PCR machine at

Lahor Research Laboratories, Benin City, Nigeria. Quick load OneTaq one-step PCR master mix 2x (New England Biolab, USA) was purchased from Inqaba Biotech, Hartfield, South Africa and used according to the manufacturer's instructions. The PCR was performed in 25 µl reaction mixture containing 12.5 µl Quick load OneTaq one-step PCR master mix (2x), 1.25 µl of each gene-specific forward and reverse primer (20 µM), 5.0 µl of nuclease free water and 5 µl of DNA template. The PCR was started immediately as follows: Initial denaturation at 94 °C for 3 minutes, denaturation at 94 °C for 30 seconds, annealing at 54 °C for 30 seconds, extension at 72 °C for 1 minute, for

35 cycles, final extension at 72 °C for 10 minutes and final hold at 4 °C forever. Ten microliters (10 µl) of the amplified PCR products were fractionated on a 1.0% agarose gel containing ethidium bromide in Tris/Borate/EDTA (TBE) Buffer. Electrophoresis was performed at 90 volts for 60 minutes. Products were visualized by Wealth Dolphin Doc UV transilluminator and photographed. Molecular weights were calculated using molecular weight standard maker.

Statistical analysis

Percentage of isolates with and without antibiotic resistance, with and without Integron genes was calculated using

$$\% = \frac{\text{Number of isolates that harbors the character} \times 100}{\text{Total number of isolates}}$$

RESULTS

Resistance pattern of *P. aeruginosa*

Resistance pattern of *P. aeruginosa* isolates against 8 antibiotics tested is shown in (Figure 1). In the present study, 62 *P. aeruginosa* isolated from 284 surgical wound infections were screened to identify antibiotic resistance strains and the presence

of *int1*, *int2* and *int3* genes. Thirty four (54.8%) isolates showed multiple drug resistance ability. On the basis of the antibiotics susceptibility results obtained, most isolates showed high resistance to Ceftazidime, Augmentin, Cefixime and gentamicin (54.8%) while resistance to ciprofloxacin was (35.5%).

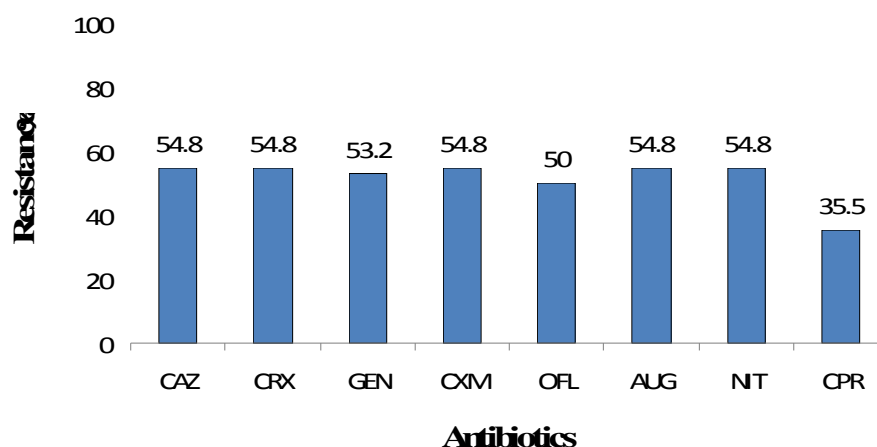


Figure 1: Antimicrobial resistance pattern of *P. aeruginosa* isolates. CAZ: Ceftazidime, CRX: Cefuroxime, GEN: Gentamicin, CXM: Cefixime, OFL: Ofloxacin, AUG: Augmentin, NIT: Nitrofurantoin, CPR: Ciprofloxacin

PCR detection of integron genes

Based on the PCR results, 16 (47.1%) *P. aeruginosa* strains harbors integron genes with integron universal primer while eighteen (52.9%) *P. aeruginosa* strains did not harbor any integron genes (Figures 2 and 3). 13 (81.3%) isolates carried *int1* gene (Figures 4 and 5), 8 (50%) isolates harbored

int2 gene (Figures 6 and 7) while 6 (37.5%) isolates carried *int3* gene (Figures 8 and 9). Integron class 1, 2 and 3 genes were detected in two *P. aeruginosa* strains. The relationship between *P. aeruginosa* strains comprising *int1* and antibiotic resistance have been previously reported and all resistant strains harbored the gene.

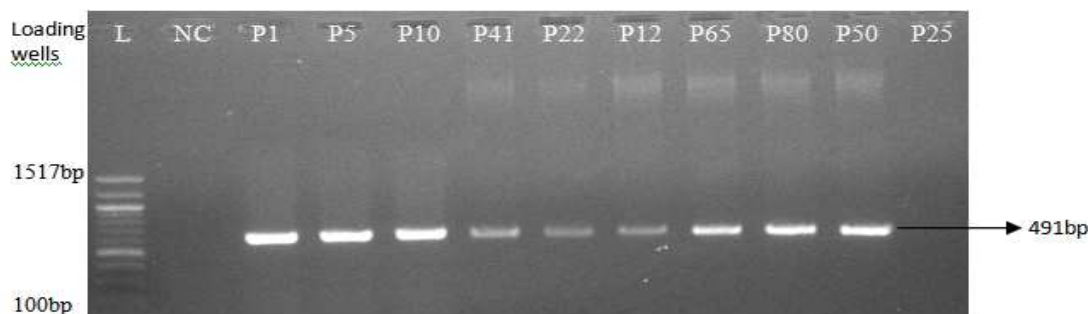


Figure 2: Molecular detection of integrase genes in genomic DNA of *Pseudomonas aeruginosa* strains using polymerase chain reaction technique.

Isolates P1 to P50 were positive with bands at 491bp while isolates P25 is negative for integrase genes. L: DNA ladder (100bp – 1517bp), NC: negative control.

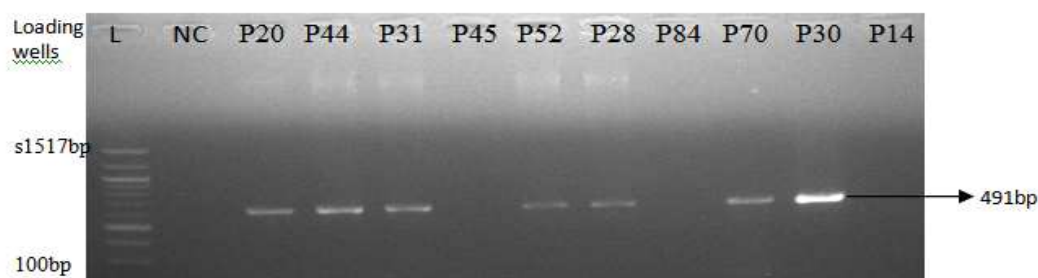


Figure 3: Molecular detection of integrase genes in genomic DNA of *Pseudomonas aeruginosa* strains from surgical wound swab using polymerase chain reaction technique.

Isolates P20, P44, P31, P52, P28, P70 and P30 are positive with band at 491bp while isolates P45, P84 and P14 were negative for integrase genes. L: DNA ladder (110bp – 1517bp), NC: negative control.

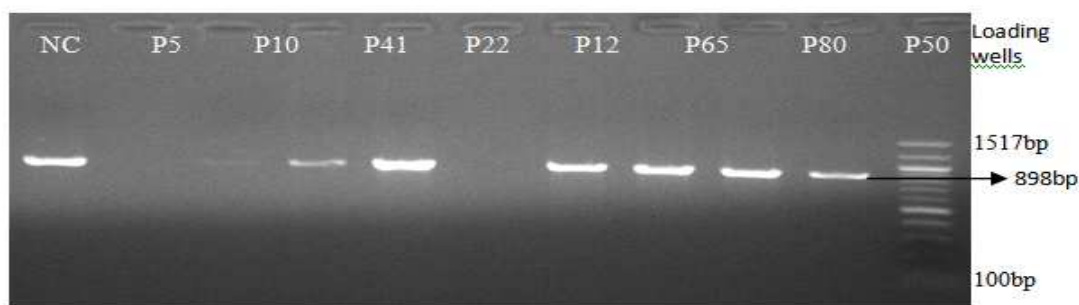


Figure 4: Molecular detection of integrase class 1 genes in genomic DNA of *Pseudomonas aeruginosa* strains.

Isolates P1, P5, P10, P41, P12, P65, P80 and P50 were positive with bands at 898bp for class 1 integron while isolates P22 is negative for integrase class 1 genes. L: DNA ladder (110bp – 1517bp), NC: negative control.

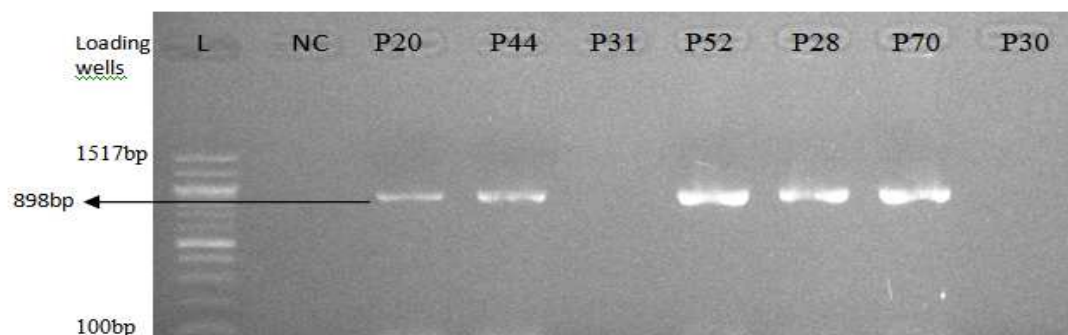


Figure 5: Molecular detection of class 1 integron genes in genomic DNA of *Pseudomonas aeruginosa* strains from surgical wound swab.

Isolates P20, P44, P52, P28 and P70 were positive with band at 898bp while isolates P31 and P30 were negative for integron class 1 genes. L: DNA ladder (110bp – 1517bp), NC: negative control.

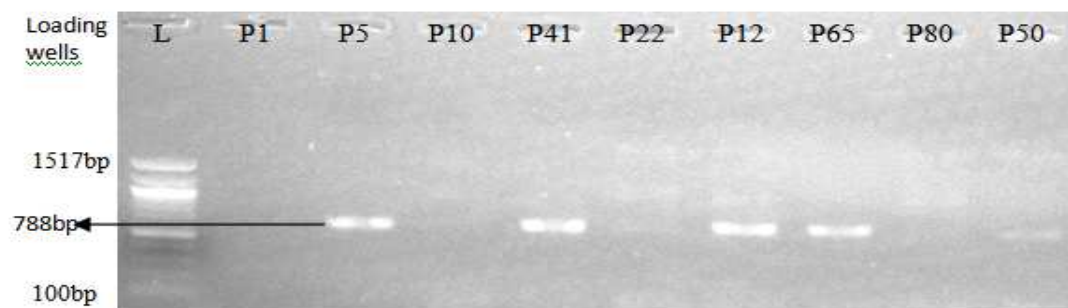


Figure 6: Molecular detection of integrase class 2 genes in genomic DNA of *Pseudomonas aeruginosa* strains.

Isolates P5, P41, P12, P65 and P50 are positive with bands at 788bp while isolates P1, P10, P22, and P80 are negative for integron class 2 genes. L: DNA ladder (110bp – 1517bp), NC: negative control.



Figure 7: Molecular detection of class 2 integron genes in genomic DNA of *Pseudomonas aeruginosa* strains from surgical wound swab.

Isolates P20, P44 and P31 was positive with band at 788bp while isolates P52, P28, P70 P30 are negative for integrase genes. L: DNA ladder (110bp – 1517bp), NC: negative control.

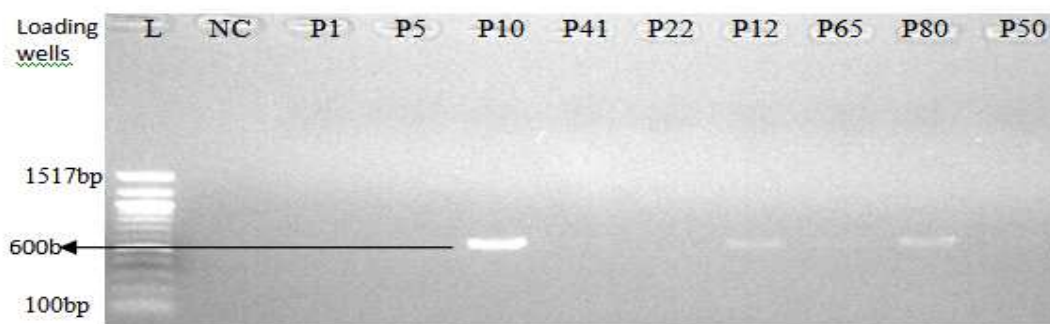


Figure 8: Molecular detection of integron class 3 genes in genomic DNA of *Pseudomonas aeruginosa* strains.

Isolates P10, P12 and P80 were positive with bands at 600bp while isolates P1, P5, P41, P22, P65 and P50 were negative for class 3 integron genes. L: DNA ladder (110bp – 1517bp), NC: negative control.

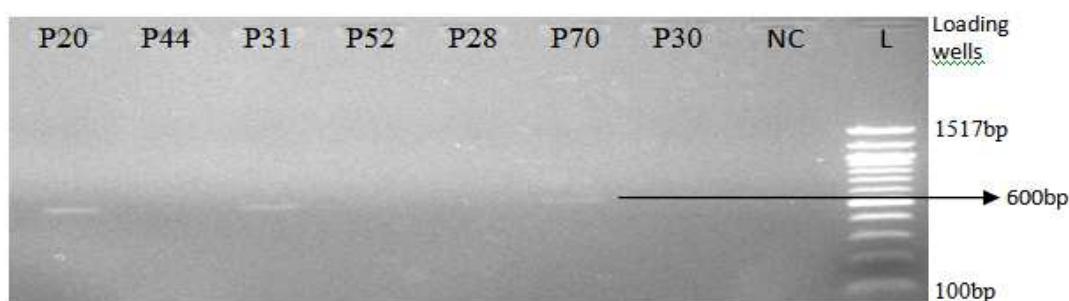


Figure 9: Molecular detection of class 3 integron genes in genomic DNA of *Pseudomonas aeruginosa* strains from surgical wound swab.

Isolates P20, P31 and P70 was positive with band at 600bp while isolates P44, P52, P28 and P30 are negative for integrase genes. L: DNA ladder (110bp – 1517bp), NC: negative control.

DISCUSSION

Antibiotic resistance genes are presently considered as emerging environmental contaminants (Siwela *et al.*, 2007). The wide spread of antibiotic resistance genes among bacteria as well as *P. aeruginosa* strains is a growing concern in the treatment of post surgical wound infections. Several antibiotics resistance genes are present as gene cassettes within bacteria integrons, which can as well be situated on mobile genetic element such as plasmids and transposons (Gu *et al.*, 2007). In this present study, according to the antibiotics susceptibility testing conducted, most of our isolates showed resistance (>50.0%) to routinely used antibiotics. Brown and Alhassan (2014) reported that antibiotic susceptibility patterns of isolated pathogenic bacteria showed that over 60% of the Gram negative enteric bacilli were resistant to at

least four of the antibiotics tested. A prior study carried out by Ehiaghe *et al.* (2016), reported high resistance of bacteria isolated from UBTH to Cefixime, Cefuroxime, Augmentin and Ofloxacin at 94%, 68%, 68% and 54% respectively while our study showed a lower resistance to same routinely used antibiotics tested against *P. aeruginosa* isolates from surgical wound infection patients in Benin City. The decrease in antibiotics resistance may be as a result of the campaign against misuse of commonly used antibiotics. Cephalosporins and Penicillins have been observed to be extremely resisted by surgical wound pathogens due to extended spectrum Beta-lactamases (Olonitola *et al.*, 2007). In the works of Eduardo *et al.* (2008) and Yah *et al.* (2010), Ceftazidime and Augmentin were mostly resistant to bacteria isolated from surgical wounds.

This is most likely to be due to the existence of Cephalosporinase and Penicillinase enzymes which can stop the action of the Beta-lactam ring composition of the antibiotics (Fontana *et al.*, 2000). In disagreement with our work, higher rate of resistance for aminoglycoside antibiotics were documented by Poonsuk *et al.* (2012). Elsewhere, Odumosu *et al.* (2013) reported a much lower rate of resistance of 22.5% and 30.11% to ceftazidime. In this present study, PCR amplification for the detection of the occurrence of three classes of integrase genes, showed that 16 *P. aeruginosa* isolates (47.1%) were found to contained integron genes out of which 13 (81.3%) isolates harbored *int1* gene. The frequency of *int1* gene in clinical strains of *P. aeruginosa* has been documented in different similar investigations from around the world and ranged from 43% to 56.3% (Yousefi *et al.*, 2010; Nikokar *et al.*, 2013; Sun *et al.*, 2014). In mentioned reports and other accepted studies, the prevalence of *int1* gene is lower when compared to this current study and this as well could be due to the trend of rapidly increasing integron positive rate among clinical strains of *P. aeruginosa* in this region.

In this study, eight (50.0%) *P. aeruginosa* which were positive for integron universal primer harbored *int2* gene. This was in contrary to the work carried out by Khosravi *et al.* (2017) who reported zero occurrence of *int2* gene among all *P. aeruginosa* screened. In the works done by Xu *et al.* (2009) and Moazami and Eftekhari (2015), the occurrence of class 2 integron was projected to be 19.5% and 2.7% respectively. Integron class 3 gene was detected in 37.5% of the isolates in this study. In contrast, Mohadeseh Zarei *et al.* (2018) reported zero occurrence of *int3* gene in their research work. Furthermore, detection of *int3* has been observed before in few Gram-negative bacteria isolates. Therefore, the surfacing and proliferation of class 3 integrons could carry a diverse gene cassette which implies that bacterial multiple drug resistance should be regarded as a

worldwide problem rather than a local problem (Shibata *et al.*, 2003).

In line with the definition of multiple drug resistance (MDR), with bacteria indicating resistance to at least three different classes of antibiotics (Rossolini and Mantengoli, 2005), all the isolates in our study were revealed to be MDR. Integrons are well-known to be associated with MDR, predominantly class 1 integrons, which are broadly spread in Gram negative bacteria including *P. aeruginosa* (Gu *et al.*, 2007). We observed high Ceftazidime, Augmentin, Cefixime and gentamicin (54.8%) resistance among *P. aeruginosa* isolates that were positive for *int1*, *int2* and *int3* genes. As stated previously, researchers had acknowledged that MDR are strongly connected with the presence of integrons, and greater part of our MDR isolates comprises of *int1* gene, however, there were some *P. aeruginosa* MDR isolates (52.9%) with no integrons (Figures 2 and 3), and hence other factors must have been responsible for the resistance of these bacteria to antibiotics.

Furthermore, our finding as well revealed the presence of *int1*, *int2* and *int3* genes in two *P. aeruginosa* (P12 and P20) isolates (Figures 4 – 9) which to the best of our knowledge is uncommon. Although the prevalence of *int2* gene is known in some reports (Goudarzi *et al.*, 2015), most *P. aeruginosa* strains in this study were found to carry *int1* gene. According to the results from our findings, it seems that the majority of antibiotics used in our research are unsuitable drugs for the treatment of *P. aeruginosa* surgical wound infections. Among them, Ciprofloxacin were the most sensitive antibiotics and may possibly be the drugs of choice.

Conclusion

In conclusion, we observed a high antibiotics resistance pattern among *P. aeruginosa* isolates in our study. The misuse and overuse of antibiotics chemotherapy may present discerning stress for the spread of MDR strains of bacterial isolates. *Int1* gene was the most abundant followed by *Int2* and *Int3* which seems to play a vital

function in MDR among bacterial isolates. Thus, routine antibiotics surveillance programs, efforts to support the correct use of antibiotics to avoid therapeutic failure is necessary for choosing the appropriate therapy and management of surgical wound infection control practices. However, more studies should be conducted to examine the cause of the appearance and prevalence of class 1, 2 and 3 integrons in one particular strain of *P. aeruginosa* in recent years. The result will assist in developing control strategies for surgical wound infections.

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