Pre and Post Plasmid Curing Effect on Pseudomonas Aeruginosa Susceptibility to Antibiotics

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Abstract: This study investigates the effect of plasmid curing on antibiotics susceptibility pattern of Pseudomonas aeruginosa clinical isolates. The study involved confirmed clinical samples of P. aeruginosa obtained from patients attending the general out-patient department of Irrua Specialist Teaching Hospital, Irrua-Nigeria. Samples with established Pseudomonas aeruginosa were subjected to antibiotic susceptibility testing before plasmid curing (group 1) and after plasmid curing (group 2) using Sodium Dodecyl Sulfate intercalating dye following previously determined standard procedures. Antibiotic resistivity and susceptibility outcomes were then determined by measuring the zone of inhibition and the pre and post cured experiments were compared. The results showed that Pseudomonas aeruginosa is 100% resistance to tetracycline, cefotaxime, ethromycin, methronidazole, nitrofuratoïn, ampicillin-clavulanate, ampicillin, amoxicillin and ampiclox. Post-plasmid curing resulted to increased susceptibility ranging from 13.3% to 92.0% as against 5.3% to 80% in the pre-plasmid cured P. aeruginosa. Based on these findings, clinical isolates of P. aeruginosa in the study area has a very high resistance rate that may be due to plasmid development or mutation of the organism and thus, the call for clinicians to carryout antibiotics susceptibility testing prior to antibiotic prescription.

Keywords: Pseudomonas aeruginosa; antibiotics susceptibility; plasmid curing; clinical isolates

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1. Introduction

Many years ago, *Pseudomonas aeruginosa* (*P. aeruginosa*) was rarely considered as a real pathogen, it was however recognized as the microorganism associated with bacteraemia in the neutropenic host in the 1970s. Nowadays, *P. aeruginosa* is the most common Gram negative opportunistic pathogen found in nosocomial infections and causing various spectra of infections especially in neutropenic, immune-compromised burns / tissue injury and cystic fibrosis patients all over the world [1, 2].

There has been increasing incidence of infections caused by multi-drug resistant organisms and has caused attention to be focused on measures of fighting resistance, foremost of which is susceptibility surveillance [3, 4]. When strains have multiple antibiotic resistance, the choice of therapy is limited, thus the tremendous therapeutic advantages afforded by the introduction of new antimicrobial agents will always be threatened by the emergence of increasingly resistant bacteria pathogen [5, 6]. Worrisome, antibiotic resistance *P. aeruginosa* has becomes a contributing factor to the re-emergence of diseases which were well-controlled before now. *P. aeruginosa* is intrinsically resistant to many structurally similar antimicrobial agents due to the low permeability of its outer membrane (1/100 of the permeability of *E. coli* outer membrane) [7], the constitutive expression of various efflux pumps with wide substrate specificity [8], and the naturally occurring chromosomal AmpC β-lactamase [9].

However, it has been reported that many antibiotic resistance genes reside in transmissible plasmids which facilitate their transfer [10]. According to Govan et al [11], plasmid-mediated resistance involving modifying enzymes is particularly associated with topical antibiotic use and with sites where high levels of antibiotics are achieved. The term plasmid was first introduced in 1952 by the American Molecular biologist, Joshua Lederberg [12]. Plasmids are covalently closed, circular, double stranded and self-replicating DNA molecule that carries few gene and are autonomous molecule that exist in cell as extra chromosomal genome. Some plasmids can be inserted into a bacterial chromosome, where they become a permanent part of the bacterial genome. They vary in size from a few genes to several hundred with the smallest plasmids contain the information necessary for replication and the larger ones code for more functions such as antibiotic resistance. Curing of plasmids is a process by which the bacterial cells lose their plasmids during cell division, these types of cells are said to be cured. Curing may occur naturally through cell division or by treating the cells with chemical and physical agents [13].

There has been accumulating evidence of altered resistance capacity in *P. aeruginosa* strains [14-16] and this could be due to loss of its resistant plasmid. Plasmid mediated resistance to various antimicrobial drugs has been demonstrated [17, 18]. In a study at Lagos University Teaching Hospital, Nigeria, resistance to gentamicin, tobramycin and carbenicillin were attributed to transferable plasmids [19]. However, the isolation and characterisation of plasmids and the occurrence and detection of AmpC L-lactamases in *P. aeruginosa* have been noted. This process entails the isolation of plasmids from the multidrug-resistant isolates, demonstrating plasmid mediated resistance by plasmid-curing and transformation experiments. This study was therefore undertaken to compare the antibiotics susceptibility pattern following pre-cured and post-cured plasmid bearing isolate of *P. aeruginosa* strains from clinical specimens.
2. **Materials and Methods**

2.1 **Materials / Equipment**

The materials and equipment used in this study includes: Bijoux bottles, test tubes, measuring cylinder, petri dishes, conical flask, weighing balance, bunsen burner, aluminium foil, innoculating wire loop, pasteur pipette, spatula, test tubes rack, cotton wool, autoclave, sterile distilled water, disinfectant, hot air oven and incubator.

2.2 **Media**

The media used comprised of Muller-Hinton agar, Nutrient agar and peptone water. Media were prepared according to manufacturer’s guide.

2.3 **Samples**

The sample study population included all patients attending the general out-patient department of Irrua Specialist Teaching Hospital (ISTH) Irrua. However, only subjects with confirmed *P. aeruginosa* infections formed the test population. Samples were collected, stored and processed using standard laboratory procedures.

The research was conducted in the Medical Microbiology Laboratory of Ambrose Alli University, Ekpoma. Over the study period, a total of seventy five *P. aeruginosa* isolates (comprising of 33 female and 42 male subjects) from one hundred and forty three (143) clinical specimens were identified and examined by standard bacteriological methods (Colonial morphology, citrate test, oxidase test, indole test) and growth at 42°C. The isolates were recovered from urine, urethral swab aspirate, wound swab, ear swab and high vaginal swab (HVS).

2.4 **Sterilization of Equipment Used**

Wire loops were sterilized by passing them through a Burnsen burner flame until it was red hot before use. While all glass wares were washed with detergent, rinsed in distilled water and sterilized at 160°C in the hot air oven for 1 hour before use.

2.5 **Identification of Isolates**

Morphological characteristics and biochemical tests were used to identify isolates.

2.6 **Plasmid curing experiment**

The curing of plasmid DNA content in *P. aeruginosa* isolates was carried out according to Lexmi et al. [20] and Winker et al. [21]. The curing agent used was Sodium Dodecyl Sulfate (SDS).-a DNA intercalating dye. In the experimental process, resistance plasmid DNA of multidrug resistant strain was cured by inoculating 10ml of nutrient broth with the test isolates and incubated for 3-4 hours to allow minimal growth of the organism. One millilitre of 10% SDS (curing agent) was added and this brought the concentration to 1% [w/v] SDS. It was then incubated for 24-48 hours at 37°C, thereafter, the antibiotic susceptibility of the cured isolates were determined as previously described.

2.7 **Antibiotic susceptibility testing**

In vitro antibiotic sensitivity of strains before curing, where determined by Kirby- Baur disc diffusion technique, and as previously described by Daini and Adesemowo [22], and standardized by the method of National Committee for Clinical Laboratory Standards [23]. The following antibiotic Gram negative muti discs were used: Ampicillin, Amoxcillin, Ampiclox, Fluzapen, Unasyn, Ampicillin-clauvulanate, Gentamycin, Tobramycyn, Streptomycin, Chloramphenicol, Tetracycline,
Cotrimoxazole, Methronidazole, Nitrofurantoin, Erythromycin, Ofloxacin, Ciprofloxacin, Pefloxacin, Ceftriazone, Cefotaxime and Cefuroxime.

The nutrient agar and Muller Hinton agar used for the sensitivity test of the isolate pre and post plasmid curing, were prepared according to the manufacturer’s instruction. Overnight broth cultures of the test isolate adjusted to $10^5$cfu/ml were plated by aseptically spreading unto Muller –Hinton agar plates. Commercially available Gram negative antibiotic sensitivity disc were placed aseptically on the inoculated nutrient medium with the aid of a sterile forcep. The plates were incubated at 37°C for 24hours [24]. The zone of inhibition was measured and recorded in mm in accordance to the NCCLS Criteria [23].

2.8 Data Analysis

Data were analysed using the Statistical Package for Socio Sciences (version 17) and where applicable the simple descriptive statistics was carried out. Results were then presented in suitable tables and charts for summarization and simplicity.

3. Results

A total of seventy-five $P. aeruginosa$ positive clinical samples from attendees of the out patients department of the Irrua Specialist teaching hospital were used for this study. Table 1 shows the distribution of these samples by age and sex. Samples from male accounted for 44.0% while female samples accounted for 56.0%. Most samples were derived from patients within the age range of 20-39 years (36; 48.0%) and the least from patients between 40-59 years (5; 6.7%). Patients less than 19 years and those above 60 years contributed 14.7% and 30.7% of the total samples.

Table 1 Age and sex distribution of the $Pseudomonas aeruginosa$ isolates

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Age Groups (Years)</th>
<th>Males (n=33; 44.0%)</th>
<th>Female (n=42; 56.0%)</th>
<th>Total (N=75; 100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0-19</td>
<td>5 (6.7)</td>
<td>6 (8)</td>
<td>11 (14.7)</td>
</tr>
<tr>
<td>2</td>
<td>20-39</td>
<td>15(20)</td>
<td>21 (28)</td>
<td>36 (48.0)</td>
</tr>
<tr>
<td>3</td>
<td>40-59</td>
<td>3(4)</td>
<td>2(2.7)</td>
<td>5 (6.7)</td>
</tr>
<tr>
<td>4</td>
<td>&gt;60</td>
<td>10(13.3)</td>
<td>13(17.3)</td>
<td>23 (30.7)</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>33(44%)</td>
<td>42(56%)</td>
<td>75(100)</td>
</tr>
</tbody>
</table>

Figure 1 presents the sources of the clinical samples from which $P. aeruginosa$ were isolated. Urine (46.6%) and wound swab (33.3%) samples were the major sources for recovering $P. aeruginosa$. Samples were also recovered from ear swab (n = 6; 8%), aspirate (n = 4; 5.3%), high viginal swab (n = 3; 4%) and urethral swab (n = 2; 2.7%).
Table 2  Pre and post plasmid curing antibiotic susceptibility pattern of *Pseudomonas aeruginosa* isolates

<table>
<thead>
<tr>
<th>ANTIBIOTICS</th>
<th>Pre- plasmid curing</th>
<th>Post- plasmid curing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S (%)</td>
<td>R (%)</td>
</tr>
<tr>
<td>PENICILLINS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0(0)</td>
<td>75(100)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>0(0)</td>
<td>75(100)</td>
</tr>
<tr>
<td>Ampiclox</td>
<td>0(0)</td>
<td>75(100)</td>
</tr>
<tr>
<td>B-LACTAMAEs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flazopen</td>
<td>16(21.3)</td>
<td>59(78.7)</td>
</tr>
<tr>
<td>Amoxicillin-clavulanate</td>
<td>0(0)</td>
<td>75(100)</td>
</tr>
<tr>
<td>Unasyn</td>
<td>12(16)</td>
<td>63(84)</td>
</tr>
<tr>
<td>AMINOLYPOSIDES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamycin</td>
<td>29(38.7)</td>
<td>46(61.3)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>40(53.3)</td>
<td>35(46.7)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>31(41.3)</td>
<td>44(58.7)</td>
</tr>
<tr>
<td>IMMIDAZOLES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methronidazole</td>
<td>0(0)</td>
<td>75(100)</td>
</tr>
<tr>
<td>Nitrofuraxia</td>
<td>0(0)</td>
<td>75(100)</td>
</tr>
<tr>
<td>MACROLIDES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethromycin</td>
<td>0(0)</td>
<td>75(100)</td>
</tr>
<tr>
<td>QUINOLONES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>20(26.7)</td>
<td>55(73.3)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>60(80)</td>
<td>15(20)</td>
</tr>
<tr>
<td>Pefloxacin</td>
<td>30(40)</td>
<td>45(60)</td>
</tr>
<tr>
<td>CEPHALOSPORINS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceftriazone</td>
<td>13(17.3)</td>
<td>62(82.7)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0(0)</td>
<td>75(100)</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>28(37.3)</td>
<td>47(62.7)</td>
</tr>
<tr>
<td>COMMONLY USED</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>4(5.3)</td>
<td>71(94.7)</td>
</tr>
<tr>
<td>Chloraamphenicol</td>
<td>4(5.3)</td>
<td>71(94.7)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0(0)</td>
<td>75(100)</td>
</tr>
</tbody>
</table>

Table 2 presents the antibiotic resistivity and susceptibility pattern of *P. aeruginosa* pre and post plasmid curing. *P. aeruginosa* showed increased susceptibility post-plasmid curing in all the antibiotics herein investigated except for the Ampicillin, Ampicillin-clavulanate, Nitrofurazone and Ethromycin where there were 100% resistivity pre and post-plasmid curing. Comparatively, penicillins that had a 100% resistance to it by *P. aeruginosa* pre plasmid curing, subsequently, showed susceptibilities ranging from 13.3% to 16.0% post plasmid curing. Prior to plasmid curing, the B-lactames antibiotics showed susceptibilities ranging from 16.0% to 21.3% but their susceptibilities range from 26.7% to 54.7% post plasmid curing. The aminoglycosides antibiotics were susceptible to *P. aeruginosa* pre-plasmid curing (38.7% to 53.3%) and post-plasmid curing (50.7% to 86.7%). For the Imidazoles group of antibiotics, there was 100% resistance by *P. aeruginosa* pre-plasmid curing but post-plasmid curing, only methronidazole was susceptible (37.3%). However, the macrolides was 100% resistance to it by *P. aeruginosa* pre and post-plasmid curing. Quinolones group of antibiotics were highly effective against *P. aeruginosa* with range from 26.7% to 80.0% pre-plasmid but range from 54.7% to 92% post-plasmid curing. Similarly, the cephalosprins showed susceptibility to *P. aeruginosa* with range from 17.3% to 37.3% pre-plasmid curing but 54.7% to 86.7% post-plasmid curing.
curing. While the commonly used antibiotic group show low susceptibility to *P. aeruginosa* during pre-plasmid curing (5.3%) this susceptibility to *P. aeruginosa* was observed to increase post-plasmid curing with range from 16.0% to 37.3%. It was shown that post-plasmid curing raises the susceptibility potential to *Pseudomonas aeruginosa* isolates above 50% in aminoglycoside’s, quinolones’ and cephalosprins groups of antibiotic (see figure 1).

![Figure 1 Susceptibility to *Pseudomonas aeruginosa* isolates pre and post-plasmid curing to the different groups of antibiotics](image)

### 4. Discussion

*Pseudomonas aeruginosa* is one of the most common nosocomial pathogen and the infections due to this organism are often difficult to treat due to antibiotic resistance. This study showed that *P. aeruginosa* is resistance to several antibiotics. In this study, 9 out of the 21 antibiotics here in studied presented 100% resistant to *P. aeruginosa* from clinical isolate. This finding showed *P. aeruginosa* to present an alarming multiple antibiotics resistivity. In accordance with this finding, resistance of *P. aeruginosa* to various antimicrobial agents has been reported by several researchers including Emori and Gaynes [25] and Yimaz *et al.* [26].

The finding of this study showed that only Ciprofloxacin (80.0%) and Streptomycin (53.3%) presented susceptibility greater than 50% with Tetracycline, Cefotaxime, Ethromycin, Methronidazole, Nitrofuratoin, Ampicillin-claavulanate, Ampicillin, Amoxicillin and Ampiclo present 100% resistance and others with susceptibility ranging from 5.3% to 41.3%. Taiwo *et al.* [27] reported *P. aeruginosa* to be sensitive in the range 70% - 94% to Ciprofloxacin and Ofloxacin and 55% - 90% to Gentamicin, Ceftriaxone, and Azithromycin in a study in Ilorin. Sule et al., [28] on the other hand in a study in Shagamu reported *P. aeruginosa* sensitivity in the range of 82.8% - 89.2% to fluoroquinolones, 61.8% - 75% to aminoglycosides and 1.7% - 46.8% to Co-trimoxazole, Ampicillin, and Tetracycline. Ogiwara *et al.* [29] in a study in Japan has reported high resistance of over 90% of the organisms to Tetracycline, Nalidixic acid, Nitrofurantoin, Cotrimoxazole and Ampicillin which is comparable to the finding in the present study. Similarly, Goniugur *et al.* [30] in a study in Turkey reported 100% resistance to Ampicillin and Penicillin and compared to our finding as well with that from Egypt where Elkholy *et al.* [31] reported a resistance of about 95% *P. aeruginosa* isolates to
Ampicillin.

This study showed that *P. aeruginosa* from clinical isolate from the study area is 100% resistant to multiple antibiotics (9 out of the 21 antibiotic used) and low susceptibility to many. Multiple antibiotics resistance, index higher than 0.2 has been said to be an indication of isolates originating from an environment where antibiotics were often used [32, 33]. The practical significance of the index may however be lost in Nigeria and other 3rd world countries where antibiotic use and abuse is rampant since the cutoff point was determined in countries with tight antibiotic control protocols. The multiple antibiotics resistance values here as reported can however be viewed as an indication of the extent of microbial exposure to antibiotics used within the community.

Analysis of the resistance pattern pre-plasmid curing showed that *P. aeruginosa* strains from clinical isolate had about 42.9% multiple antibiotics resistance with only 9.5% showing susceptibility above 50% of the 21 antibiotic herein investigated. On the other hand, post-plasmid curing *P. aeruginosa* from clinical isolate presented about 19.1% multiple antibiotics resistance with about 47.6% showing susceptibility above 50% of the 21 antibiotic herein investigated. The comparable high susceptibility post-plasmid curing over pre-plasmid curing indicates that *P. aeruginosa* strains from clinical isolate in the study area harboured plasmids and as such has developed plasmid mediated resistance. This is based on the fact that the high levels of resistance to antibiotics in this study, has been ascribed to the presence of plasmids [34-36].

In another line of thought, the higher susceptibility with post-plasmid curing compared to pre-plasmid curing showed plasmid can inhibit antibiotics activity. The findings therefore showed that plasmid may be involved in the high resistance to antibiotics by the *P. aeruginosa* strains from clinical isolates. This assertion is in line, considering that plasmid-mediated resistance to various antimicrobial drugs has been demonstrated [17, 18]. In a study at LUTH by Rotimi *et al.* [19], resistance to Gentamicin, Tobramycin and Carbenicillin were attributed to transferable plasmids. In another study in Greece, plasmids isolated from multi-resistant *P. aeruginosa* strains were found to encode high level resistance to Gentamicin and Tobramycin [37]. In a few cases of outbreaks in Korea, Japan and Turkey, plasmids encoding potent β-lactamases together with aminoglycoside-modifying enzymes were disseminated among *P. aeruginosa* strains rendering control even more difficult [8].

By implication, the study suggests *P. aeruginosa* strains from clinical isolate in the study area has mutated. Plasmid-mediated resistance involving modifying enzymes is particularly associated with topical antibiotic use and with sites where high levels of antibiotics are achieved as reported by Govan *et al.* [11]. Moreover, it is known that many antibiotic resistance genes reside in plasmids which facilitate there transfer [38] and thus, the suggestion that *P. aeruginosa* strains from clinical isolate in the study area may have develop a resistant gene. Acquisition of mobile genetic elements is known to be the main mechanism for short term accumulation of resistance determinants in bacterial genomes [39].

5. Conclusion

It is clear from the results of this study that *P. aeruginosa* from clinical isolates in the study area has a very high resistance rate to most antibiotics and the commonly used antibiotics. In fact, *P. aeruginosa* presented 100% resistivity to tetracycline, Cefotaxime, Ethromycin, Methronidazole, Nitrofuratoin, Ampicillin-clavulanate, Ampicillin, Amoxicillin and Ampiclox. Although there were significant increases in *P. aeruginosa* susceptibility to most antibiotics post-plasmid curing, however, Ampicillin,
Ampicillin-clavulanate, Nitrofuratoin and Ethromycin were still 100% resistant to *P. aeruginosa* even after plasmid curing. The increased susceptibility to antibiotics by *P. aeruginosa* post-plasmid curing as compared to pre-plasmid curing suggests that the organism might have developed plasmid or a mutant. If this is the case, the reason no doubt is not far from inappropriate drug prescription and antibiotic misused that leads to the development and acquisition of resistance genes by bacteria. In this regard, health personnel needs to be aware of the prevailing antimicrobial activity pattern of at least, the locally available antibiotics against *P. aeruginosa* so as to make the right decision in the absence of a comprehensive antimicrobial susceptibility report. The findings of this study however buttress the need for proper laboratory diagnosis and susceptibility testing before antibiotic prescription.

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