

Plasmid Profile of Multi-Drug Resistant Phenotypes of *Pseudomonas aeruginosa* Isolated among Patients with Indwelling Catheter in Northeastern Nigeria

ABSTRACT

Aims: This study was carried out to analyze the plasmid profile of multidrug resistant *Pseudomonas aeruginosa* isolated among catheterized patients attending University of Maiduguri Teaching Hospital. **Place of Study:** Department of Medical Microbiology (Laboratory Section), University of Maiduguri Teaching Hospital and Department of Biological Sciences, Abubakar Tafawa Balewa University, Nigeria

Methodology: 244 samples (catheter tip, urethral swab, urine) were collected from catheterized patients and investigated via microscopy and culture on Blood agar and MacConkey agar. Suspect *Pseudomonas aeruginosa* isolates were further confirmed using biochemical tests. Kirby bauer disc diffusion test was used to determine the antimicrobial susceptibility pattern. Isolates confirmed to be multidrug resistant (MDR) were subjected to plasmid profile analysis using agarose gel electrophoresis.

Results: 21 yielded *Pseudomonas aeruginosa* which gives a recovery rate of 8.6%. A significant proportion was isolated from catheter tip samples collected from male patients (33.33%). The association between sex of patient and sample type in the isolation of *Pseudomonas aeruginosa* was statistically significant ($X^2 = 10.76$, $df = 2$, $P < .01$). Isolates were most-sensitive/least-resistant to Ofloxacin and Ampiclox, and least-sensitive/most-resistant to Penicillin. All isolates identified were multi-drug resistant (MDR) with an average resistance rate of 3.28 antimicrobials per isolate. Plasmid analysis revealed that 57.14% of isolates possessed similar plasmid with a DNA fragment size of 300bp and a molecular weight of 31ng/10 μ l.

Conclusion: We establish a very high rate of multidrug resistance among *Pseudomonas aeruginosa* isolates. Plasmid profile analysis of MDR *Pseudomonas aeruginosa* observed revealed a high plasmid prevalence rate and since most isolates cannot express the resistance marker after plasmid curing, we conclude that this is indicative of the plasmidial origin of such a marker.

Keywords: [*Pseudomonas aeruginosa*, Multi-drug resistance, Plasmid profile, Antimicrobial susceptibility]

1. INTRODUCTION

Pseudomonas aeruginosa is an aerobic, non-fermenting Gram-negative bacillus, which is most commonly involved in opportunistic infections mostly in the nosocomial setting (Rossellini *et al.*, 2005; Tam *et al.*, 2010). It is an ubiquitous organism frequently isolated from clinical specimens and accounts for a significant proportion of nosocomial infections (Blanc *et al.*, 1998). Naturally, this organism is endowed with weak pathogenic potential. However, its profound ability to survive on inert materials, minimal nutritional requirement, tolerance to a wide variety of physical conditions and its relative resistance to several unrelated antimicrobial agents and antiseptics, contribute enormously to its ecological success and its role as an effective opportunistic pathogen (Gales *et al.*, 2001).

Nosocomial isolates of *Pseudomonas aeruginosa* exhibit high rate of resistance to antibiotics and are often multidrug resistant (Brisse *et al.*, 2000). Thus, outbreaks due to multi-drug resistant *Pseudomonas aeruginosa* have been reported, especially in nosocomial settings such as intensive care units (ICUs) (Smith *et al.*, 2012). *Pseudomonas aeruginosa* remains a classic opportunistic pathogen due to innate as well as acquired resistance conferred by plasmids and an armoury of putative virulence factors (Shadid and Malik, 2004).

A plasmid is a self-replicating extrachromosomal genetic element that is not essential for normal bacterial growth but houses genes for various determinants such as antibiotic resistance and toxin production. Plasmids can be supercoiled, circular or linear and are transferred between bacteria of the same or different genera. Plasmids have been reported as the major mechanism for the spread of antibiotic resistant genes in bacterial populations (Fang *et al.*, 2008). Since plasmids are transferred so readily among bacteria, and some can replicate in various species, a single plasmid has been observed in several bacterial species during a number of infectious disease outbreaks. These outbreaks are sometimes called plasmid epidemics.

Plasmids have been shown to confer advantageous traits upon *P. aeruginosa* clinical isolates. It has been commonly seen that antibiotic resistance in *P. aeruginosa* was correlated with the presence of IncP group conjugative plasmids. Moreover, many resistance plasmids are being detected in *P. aeruginosa* (Strateva and Yordanov, 2009). Many of the resistance genes are found embedded in or associated with mobile elements such as transposons, integrons, and IS elements. In addition, apart from the genomic island-encoded virulence, *P. aeruginosa* plasmid-mediated pathogenicity was revealed. Plasmids in *P. aeruginosa* are also involved in other advantageous traits (Yeldho *et al.*, 2011).

The ability of a genetic marker to be transferred from one bacterium to another through conjugation or transformation provides a good presumptive evidence for the involvement of plasmid, particularly if the frequency of transfer is high. Moreover, loss of certain genetic markers as a result of treatment of bacterial cell to plasmid curing agents further suggests for the plasmidial nature of the marker (Mesas *et al.*, 2004). Here, we try to evaluate the plasmid profile of multi-drug resistant *Pseudomonas aeruginosa* isolated among catheterized patients.

2. MATERIAL AND METHODS

2.1 Study Area and Population

This study was carried out at the University of Maiduguri Teaching Hospital (UMTH), Maiduguri. Maiduguri is a city located in the north-eastern part of Nigeria and lies within latitude 11.50°N and longitude 30.50°E in the sudano-sahelian savanna zone with a dense population that are mostly crop farmers, fishermen, herdsman and traders. It is a city with a rich cultural heritage and a home to the kanem Borno Empire (Udoh, 1978). The target population for the study include patients that attended the clinics and those admitted in the wards of the hospital (Outpatients and Inpatients).

2.1.1 Inclusion criteria

All patients that were on catheter or have used catheter during the period of study.

2.1.2 Exclusion criteria

All patients that are not on catheter or have not used catheter during the period of study.

2.2 Sample Size Determination

The sample size was determined using the formula described by Fischer *et al.*, (1998).

$$n = N/1 + N(e)^2 = 250/1 + 250(0.01)^2 = 244$$

Where:-

n = the desired sample size (when population < 10,000)

N = population number of catheterized patients given as 250.

e = degree of accuracy desired (0.01 which corresponds to 99% confidence level).

2.3 Bacterial Isolation and Identification

Two hundred and forty four (244) samples (catheter tips, urethral swabs, urine) were collected from catheterized patients attending the various wards and clinics of the University of Maiduguri Teaching Hospital (UMTH). Catheter tips (approximately 4 cm) were cut aseptically and placed into a sterile container containing about 5ml sterile normal saline, urethral swabs were collected using sterile swab stick and mid-stream urine samples were collected in a sterile universal container and transported to the laboratory for analysis. Samples were inoculated on Blood agar and MacConkey agar and incubated at 37°C for 24hrs. Colonies suspected to be *Pseudomonas aeruginosa* was further confirmed by Motility test, Gram stain, citrate test, catalase test, and oxidase test, Glucose test, and pyocin pigmentation test (Cheesbrough, 2006).

83 2.4 Antibacterial Sensitivity Testing

84 Antibiotic susceptibility testing was determined using the Kirby-Bauer disc diffusion method as
85 described by Akubueyi *et al.* (2011) and interpreted in accordance with the Clinical and Scientific
86 Laboratory Institute guidelines (CSLI, 2017). The antibiotics tested were Amoxicillin (20µg/ml),
87 Gentamicin (10µg/ml), Augmentin (30µg/ml), Chloramphenicol (30µg/ml), Streptomycin (30µg/ml),
88 Ofloxacin (30µg/ml), Pefloxacin (10µg/ml), Ciprofloxacin (10µg/ml), Cefuraxime (30µg/ml), Septrin
89 (30µg/ml) Penicillin (30µg/ml), Rifampicin (20µg/ml), Erythromycin (30µg/ml), Ampiclox (20µg/ml),
90 Levofloxacin (20µg/ml) (Fondoz laboratories, Nigeria; Oxoid Limited, UK).

91 2.5 Determination of Multiple Antibiotic Resistance (MAR) Index

92 The Multiple Antibiotic Resistance (MAR) index was determined for *P. aeruginosa* isolates by dividing
93 the number of antibiotics to which the isolate was resistant by the total number of antibiotics tested
94 (Paul *et al.*, (1997); Olayinka *et al.*, (2004)).

$$95 \text{ MAR index} = \frac{\text{Number of antibiotics isolate is resistant to}}{\text{Total number of antibiotics tested}}$$

97 2.6 Plasmid DNA Extraction and Gel Electrophoresis

98 Plasmid isolation was performed using the alkaline lysis method described by He, F. (2011). After
99 isolation of plasmid DNA, a horizontal agarose gel electrophoresis was carried out based on the
100 method described by (Meyers *et al.*, 1996).

101 2.7 Plasmid Curing

102 Suspected Plasmid harboring isolates identified were subjected to plasmid curing using the
103 modifications of Olukoya and Oni (1990). Resistance markers expressed after curing were regarded
104 as being chromosome-mediated while those that did not express resistance were regarded as
105 plasmid mediated.

106 2.8 Data Analysis

107 Data generated were analyzed using Statistical Package for Social Sciences (SPSS, version 16.0).
108 Data were presented as frequencies and percentages. Chi-square was used and evaluations were
109 carried out at 99% confidence level and $P < 0.01$ was considered as statistically significant.

110 3. RESULTS AND DISCUSSION

111 Twenty one (21) out of the two hundred and forty four (244) samples processed yielded *P.*
112 *aeruginosa*. This accounted for a prevalence rate of 8.61%, which was higher among male patients
113 (61.90%) than female patients (38.09%). Similar study conducted by Olayinka *et al.* (2004) reported a
114 prevalence rate of 10.5%. Higher rate of 23.2% was reported by Umar *et al.* (2016) in the study area.
115 It has been reported that *Pseudomonas aeruginosa* is the second leading cause of gram-negative
116 nosocomial infection (Carmeli *et al.*, 1999) and as such, its detection (even in negligible proportion)
117 should be a source of concern.

118 Significant bacterial yield was observed among catheter tip samples (42.85%) and the least was
119 observed among urethral swab samples (23.81%). On sex versus sample type basis, rate of isolation
120 was highest among catheter tip samples collected from male patients (33.33%). It has been shown
121 that the use of indwelling catheters create an inherent risk for infection (Wong, 1983).

122 *Pseudomonas aeruginosa* especially endangers vulnerable hosts such as immunocompromised
123 persons or patients with indwelling medical devices such as catheters. It is also one of the main
124 causative agents of catheter-related nosocomial urinary tract infections (Mittal *et al.*, 2009; Hola *et al.*,
125 2010). The insertion of a catheter (either urinary or bloodstream) further increases the risk of blood-
126 stream infection in already fragile patients (Trautner and Darouiche, 2004). However, the association
127 between sex of patient and sample type in the isolation of *Pseudomonas aeruginosa* was statistically
128 significant ($X^2 = 10.76$, $df = 2$, $P < .01$). (Table 1).

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Table 1: Percentage rate of occurrence of *P. aeruginosa* among patients attending University of Maiduguri teaching hospital

Sex	Sample Types (n=21)			Total (%)
	Catheter tips (%)	Urethral swab (%)	Urine (%)	
Male	7 (33.33)	5 (23.81)	1 (4.76)	13 (61.90)
Female	2 (9.52)	0 (0.00)	6 (28.57)	8 (38.09)
Total	9 (42.85)	5 (23.81)	7 (33.33)	21 (100)

($\chi^2 = 10.76$, $df=2$, $P < .01$)

High frequency of infection was found among patients within the age-group of 70-79years (38.09%) and least among patients of 0-9years and 10-19years (4.76% respectively). A significant infection rate was observed among urine samples collected from patients within the age group of 70-79years (19.05%) on age versus sample type basis. However, the association between age of patients and the rate of isolation of *P. aeruginosa* among positive sample types was statistically not significant ($\chi^2 = 18.65$, $df = 14$, $P < .01$) (Table 2).

Table 2: Relationship between age of patients and the rate of isolation of *P. aeruginosa* among positive sample types

Age group (years)	Sample Types (n=21)			Total (%)
	Catheter tips (%)	Urethral swab (%)	Urine (%)	
0-9	0 (0.00)	0 (0.00)	1 (4.76)	1 (4.76)
10-19	0 (0.00)	1 (4.76)	0 (0.00)	1 (4.76)
20-29	1 (4.76)	1 (4.76)	0 (0.00)	2 (9.52)
30-39	2 (9.52)	0 (0.00)	0 (0.00)	2 (9.52)
40-49	0 (0.00)	1 (4.76)	2 (9.52)	3 (14.29)
50-59	1 (4.76)	1 (4.76)	0 (0.00)	2 (9.52)
60-69	3 (14.29)	1 (4.76)	0 (0.00)	4 (19.05)
70-79	2 (9.52)	0 (0.00)	4 (19.05)	8 (38.09)
Total	9 (42.86)	5 (23.81)	7 (33.33)	21 (100)

($\chi^2 = 18.65$, $df = 14$, $P < .01$)

Antimicrobial susceptibility test revealed that isolates were most sensitive/least resistant towards Ofloxacin and Ampiclox, and least sensitive/most resistant to Penicillin. Resistance was shown across all families of antimicrobial drugs tested but was more pronounced against Beta-lactam antibiotics (Figure 1).

It has been reported that catheter associated urinary tract infections (CAUTIs) comprise perhaps the largest institutional reservoir of nosocomial antibiotic resistant pathogens (Maki, 1981). This is quite worrisome because of the fact that catheterized patients are mostly immunocompromised and drugs such as tetracycline, which inhibit bacterial growth, usually depend on an active immune system for onward clearance of inhibited bacteria. As such, multidrug resistant *P. aeruginosa* poses a dire clinical challenge in terms of patient therapy, and infection control and prevention within the hospital environment. It is also suggested that over-enthusiastic use of broad spectrum antibiotics can pave way to a rapid emergence of resistance among various bacterial species.

Ibrahim *et al.*, (2000) reported that *Pseudomonas aeruginosa* is one of the main organisms responsible for drug-resistant nosocomial infections, and is one of the leading causes of bacteraemia and pneumonia in hospitalised patients. In addition to being intrinsically resistant to several

antimicrobial agents, *P. aeruginosa* acquires resistance readily to conventional anti-pseudomonal antibiotics (i.e., anti-pseudomonal penicillins, ceftazidime, fourth-generation cephalosporins, aztreonam, carbapenems and ciprofloxacin) following prolonged use of these antibiotics in hospitalised patients (Troillet *et al.*, 1997).

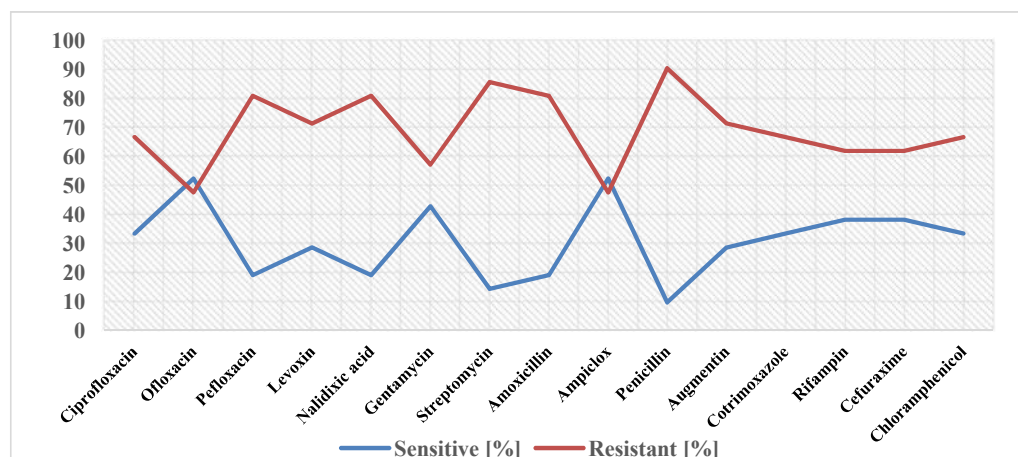


Figure 1: Antimicrobial susceptibility pattern of *P. aeruginosa* isolated from catheterized patients attending University of Maiduguri Teaching Hospital

All isolates observed in this study were multi drug resistant (100%), with significant proportion recovered from catheter tip samples (42.86%) compared to urine (33.33%) and urethral swab samples (23.81%) being the least (Table 3).

Table 3: Distribution of multidrug resistant (MDR) *P. aeruginosa* based on the sample types collected from patients examined

MDR <i>P. aeruginosa</i>	Sample Types (n=21)			Total (%)
	Catheter Tip (%)	Urethral Swab (%)	Urine (%)	
Positive	9 (42.86)	5 (23.81)	7 (33.33)	21 (100)
Negative	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Total (%)	9 (42.86)	5 (23.81)	7 (33.33)	21 (100)

MDR: Multidrug Resistant

Isolates were resistant to an average of 3.28 antimicrobials per isolate. Three (3) isolates were resistant to thirteen (13) antimicrobial drugs and eight (8) were resistant to twelve (12) drugs tested, giving rise to a multiple antibiotic resistance indices of 0.9 and 0.8 respectively (Table 4). Multidrug resistance in *P. aeruginosa* results from the bacterium's notable inherent antibiotic resistance, in addition to its ability to acquire and harbor diverse resistance determinants (through plasmids and integrons). Low outer membrane permeability in combination with multidrug efflux systems account for its intrinsic mechanisms of resistance. The resistance-nodulation-cell division (RND) family of transporters is responsible for a significant portion of clinically relevant drug resistance among Gram-negative bacteria and facilitates active efflux of multiple antimicrobial substrates (Li and Nikaido, 2004). Additional resistance mechanisms in *P. aeruginosa* include enzyme production and target mutations. Expression of aminoglycoside-modifying enzymes (acetyltransferases, nucleotidyltransferases and phosphotransferases), mediating aminoglycoside resistance are common (Kotra *et al.*, 2000).

Table 4: Multiple Antibiotic Resistance (MAR) and multiple resistance indices of *P. aeruginosa* isolates.

No. of antimicrobial agents to which isolates were resistant	No. of isolates with MAR (n=21)	MAR Index
6	2	0.4
8	1	0.5
9	2	0.6
10	2	0.7
11	3	0.7
12	8	0.8
13	3	0.9

MAR: Multiple Antibiotic Resistance

The plasmid analysis revealed detectable plasmids in 12 (57.14%) out of the 21 multi-drug resistant *Pseudomonas aeruginosa* isolates. Nine of the isolates possessed no plasmids while the 12 isolates possessed similar plasmids with a DNA fragment size of 300base pairs and a molecular weight of 31ng/10µl (Figure 2). Similar findings elsewhere reported a plasmid prevalence rate of 36.4% (Akingbade *et al.*, 2012). Plasmids are mobile genetic elements and can also facilitate the dispersal of resistance genes among the bacterial population and can also serve as vehicle for other resistance mechanisms (Odumoso *et al.*, 2016). The mobility characteristic of an extrachromosomal DNA means that plasmids can be easily acquired or lost, in both in vivo and in vitro conditions (Hartstein *et al.*, 1995). As a result, the transfer of resistance plasmids among *Pseudomonas aeruginosa* in a nosocomial setting is of high clinical significance.

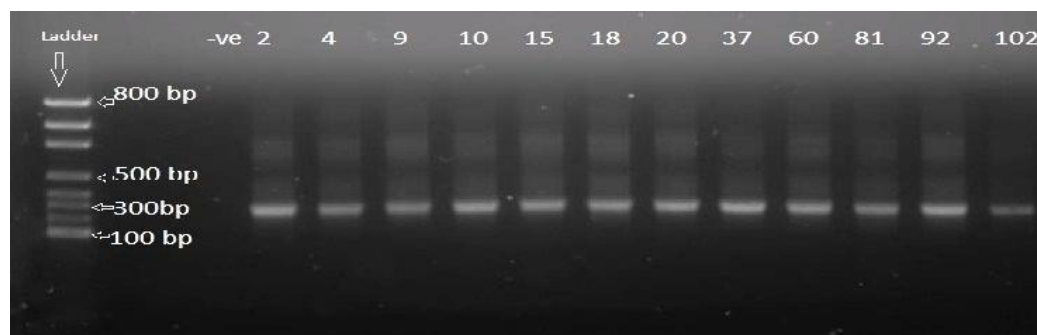
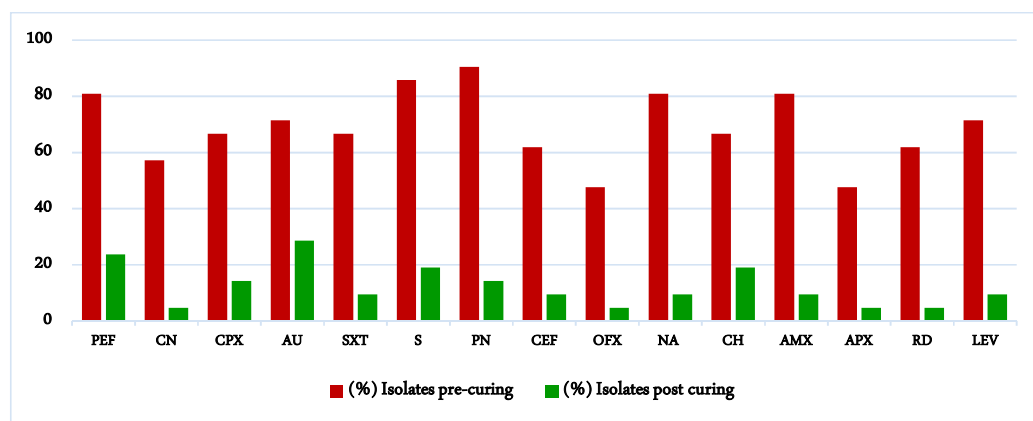


Figure 2: Plasmid profile of the multi-drug resistant isolates of *Pseudomonas aeruginosa*.

A significant proportion of isolates with detectable plasmids lost their plasmids after curing. At Antibioqram post curing stage, it was observed that some isolates that were resistant to a particular antimicrobial agent during pre-curing stage became susceptible after curing. For instance, 90.48% of isolates were resistant to ampicillin but only 4.29% showed resistance after curing (Figure 3). That means 86.19% of same isolates have lost their ability to express the resistance trait against the antimicrobial drug. This is due to the fact that the plasmid (harboring the multidrug resistance gene) had been denatured or its replication inhibited by the sodium dodecyl sulphate treatment, which was used as the curing agent.



Key: Pefloxacin=PEF, Gentamycin=CN, Ciprofloxacin= CPX, Augmentin= AU, Cotrimoxazole SXT, Streptomycin=S, Penicillin= PN, Cefuraxime= CEF, Ofloxacin= OFX, Nalidixic acid= NA, Chloramphenicol- CH, Amoxicillin= AMX, Ampiclox= APX, Rifampin= RD, Levofloxacin=LEV

Figure 3: Plasmid curing analysis of multidrug resistant *P. aeruginosa* isolated among catheterized patients

4. CONCLUSION

In conclusion, we establish that *Pseudomonas aeruginosa* isolates recovered were completely multidrug resistant. Plasmid profile analysis revealed a high plasmid prevalence and most isolates cannot express the resistance marker after plasmid curing, which indicates the plasmidial origin of the resistance marker.

CONSENT

All authors declare that informed consent was obtained from the patients (or other approved parties) for sample and data collection.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the ethics committee of the University of Maiduguri Teaching Hospital, Nigeria.

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