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

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Authors’ contributions

This work was carried out in collaboration between all authors. Authors AMU and UAF designed the study, wrote the protocol, performed the statistical analysis and wrote the first draft of the manuscript. Author MMI managed the analyses of the study, managed the literature searches and wrote the final draft of the manuscript. Authors IMT, HBA and AA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

**Aims:** This study was carried out to analyze the plasmid profile of multidrug resistant *Pseudomonas aeruginosa* isolated among catheterized patients attending the University of Maiduguri Teaching Hospital.

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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 [1,2]. It is an ubiquitous organism frequently isolated from clinical specimens and accounts for a significant proportion of nosocomial infections [3]. 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 [4].

Nosocomial isolates of Pseudomonas aeruginosa exhibit high rate of resistance to antibiotics and are often multidrug resistant [5]. Thus, outbreaks due to multi-drug resistant Pseudomonas aeruginosa have been reported, especially in nosocomial settings such as intensive care units (ICUs) [6]. 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 [7].

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 [8]. 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 [9]. 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 [10].

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 [11]. Here, we try to evaluate the plasmid profile of multi-drug resistant *Pseudomonas aeruginosa* isolated among catheterized patients.

2. MATERIALS 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, herdsmen and traders. It is a city with a rich cultural heritage and a home to the Kanem Borno Empire [12]. The target population for the study include patients that attended the clinics and those admitted to the wards of the hospital (Outpatients and Inpatients).

2.1.1 Inclusion criteria

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

2.1.2 Exclusion criteria

All patients who 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 Fisher [13].

\[ n = \frac{N}{1 + N(e)^2} = \frac{250}{1 + 250(0.01)^2} = 244 \]

Where:

- \( n \) = The desired sample size (when population<10,000)
- \( N \) = Population number of catheterised 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), Nigeria. Catheter tips (approximately 4 cm) were cut aseptically and placed into a sterile container containing about 5 ml 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 24 hrs. 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 [14].

2.4 Antibacterial Sensitivity Testing

Antibiotic susceptibility testing was determined using the Kirby-Bauer disc diffusion method as described by Akubuenyi et al. [15] and interpreted in accordance with the Clinical and Scientific Laboratory Institute guidelines [16]. The antibiotics tested were Amoxicillin (20 μg/ml), Gentamicin (10 μg/ml), Augmentin (30 μg/ml), Chloramphenicol (30 μg/ml), Streptomycin (30 μg/ml), Ofloxacin (30 μg/ml), Pefloxacin (10 μg/ml), Ciprofloxacin (10 μg/ml), Cefuraxime (30 μg/ml), Septrin (30 μg/ml) Penicillin (30 μg/ml), Rifampicin (20 μg/ml), Erythromycin (30 μg/ml), Ampiclox (20 μg/ml), Levofloxacin (20 μg/ml) (Fondoz laboratories, Nigeria; Oxoid Limited, UK).

2.5 Determination of Multiple Antibiotic Resistance (MAR) Index

The Multiple Antibiotic Resistance (MAR) index was determined for *P. aeruginosa* isolates by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics tested [17,18].

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

2.6 Plasmid DNA Extraction and Gel Electrophoresis

Plasmid isolation was performed using the alkaline lysis method described by He [19]. After isolation of plasmid DNA, a horizontal agarose
gel electrophoresis was carried out based on the method described by Meyer et al. [20].

2.7 Plasmid Curing

Suspected Plasmid harbouring isolates identified were subjected to plasmid curing using the modifications of Olukoya and Oni [21]. Resistance markers expressed after curing were regarded as being chromosome-mediated while those that did not express resistance were regarded as plasmid mediated.

2.8 Data Analysis

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

3. RESULTS AND DISCUSSION

Twenty one (21) out of the two hundred and forty four (244) samples processed yielded P. aeruginosa. This accounted for a prevalence rate of 8.61%, which was higher among male patients (61.90%) than female patients (38.09%). A similar study conducted by Olayinka et al. [18] reported a prevalence rate of 10.5%. Higher rate of 23.2% was reported by Umar et al. [22] in the study area. It has been reported that Pseudomonas aeruginosa is the second leading cause of gram-negative nosocomial infection [23] and as such, its detection (even in negligible proportion) should be a source of concern.

The significant bacterial yield was observed among catheter tip samples (42.85%) and the least was observed among urethral swab samples (23.81%). On sex versus sample type basis, rate of isolation was highest among catheter tip samples collected from male patients (33.33%). It has been shown that the use of indwelling catheters creates an inherent risk for infection [24].

Pseudomonas aeruginosa especially endangers vulnerable hosts such as immunocompromised persons or patients with indwelling medical devices such as catheters. It is also one of the main causative agents of catheter-related nosocomial urinary tract infections [25,26]. The insertion of a catheter (either urinary or bloodstream) further increases the risk of blood-stream infection in already fragile patients [27]. However, the association between sex of patient and sample type in the isolation of Pseudomonas aeruginosa was statistically significant ($X^2 = 10.76$, df = 2, $P <.01$) (Table 1).

High frequency of infection was found among patients within the age-group of 70-79 years (38.09%) and least among patients of 0-9 years and 10-19 years (4.76% respectively). A significant infection rate was observed among urine samples collected from patients within the age group of 70-79 years (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 ($X^2 = 18.65$, df = 14, $P <.01$) (Table 2).

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 (Fig. 1).

It has been reported that catheter associated urinary tract infections (CAUTIs) comprise perhaps the largest institutional reservoir of nosocomial antibiotic resistant pathogens [28]. 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.

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 [29]. 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 [30]. 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).

| Sex    | Catheter tips (%) | Urethral swab (%) | Urine (%) | Total (%) |
|--------|-------------------|-------------------|-----------|-----------|
| 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)  |

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

| Age group (years) | Sample types (n=21) | Total (%) |
|------------------|---------------------|-----------|
| Catheter tips (%)| Urethral swab (%)   | Urine (%) |
| 0-9              | 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)  | 2 (9.52) | 3 (14.29) |
| 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)  |

\(X^2 = 18.85, df = 14, P < .01\)

Fig. 1. Antimicrobial susceptibility pattern of *P. aeruginosa* isolated from catheterised patients attending University of Maiduguri Teaching Hospital
Table 3. Distribution of multidrug resistant (MDR) *P. aeruginosa* based on the sample types collected from patients examined

| MDR *P. aeruginosa* | 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 harbour 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 [31]. Additional resistance mechanisms in *P. aeruginosa* include enzyme production and target mutations. Expression of aminoglycoside-modifying enzymes (acetyltransferases, nucleotidytransferases and phosphotransferases), mediating aminoglycoside resistance are common [32].

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 300 base pairs and a molecular weight of 31 ng/10 µl (Fig. 2). Similar findings elsewhere reported a plasmid prevalence rate of 36.4% [33]. 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 [34]. The mobility characteristic of an extrachromosomal DNA means that plasmids can be easily acquired or lost, in both in vivo and in vitro conditions [35]. As a result, the transfer of resistance plasmids among *Pseudomonas aeruginosa* in a nosocomial setting is of high clinical significance.

A significant proportion of isolates with detectable plasmids lost their plasmids after curing. At Antibiogram 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 (Fig. 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 (harbouring the multidrug resistance gene) had been denatured or its replication inhibited by the sodium dodecyl sulphate treatment, which was used as the curing agent.

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
Umoru et al.; IJPR, 1(2): 1-9, 2018; Article no.IJPR.44438

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

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

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

**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.

**COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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