Antibiotic Susceptibility Pattern and Plasmid Profiles of *Pseudomonas aeruginosa* Isolated from Some Hospital Patients in Benin City, Nigeria.

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Abstract: *Pseudomonas aeruginosa*, a Gram negative rod is one of the most problematic bacterial pathogens due to its ability to readily develop resistance to many antibiotics using mechanisms like biofilm formation, production of β-lactamases, and multi-drug resistance efflux pumps. This study was aimed at investigating the resistance patterns and plasmid profiles of *Pseudomonas aeruginosa* isolated from clinical specimens. Forty (40) mid-stream urine specimens and sixty- one (61) swabs were aseptically obtained from consenting volunteers after approval from the hospital ethical committee. Specimens were inoculated on blood agar and MacConkey agar and incubated at 37°C for 24hrs. Colonies growing on media were gram – stained and identified using standard identification procedures. Antibiotic susceptibility tests were performed using the Kirby-Bauer disc diffusion technique, and results were interpreted following the Clinical and Laboratory Standard Institute's guidelines. Multi-drug resistant isolates were screened for the presence of plasmids using 10% sodium deodecyl sulphate while plasmid DNA was extracted using the technique of Bimboim and Doly and electrophoresed on a 0.8% agarose gel. Results showed that 32 (32%) of the 101 specimens yielded *Pseudomonas aeruginosa,* with 13(40.6%) of these showing multi-drug resistance phenotype. Resistance to the tested antibiotics was in the following decreasing order: Nalidixic acid and Ceporex (92.3%), Ampicillin (76.9%), Augmentin and Gentamicin (46.2%), Septrin (38.5%), Ciprofloxacin and Reflacine (30.8%), and Streptomycin (15.4%). Eleven (85%) of the resistant isolates harbored plasmids with bands ranging from 150bp to 300bp. This study has shown that plasmid mediated multi-drug resistance by *Pseudomonas aeruginosa* is rife in this locality. There is therefore urgent need for relevant health providers to initiate concerted strategies at monitoring prescribing habits of clinicians, the diagnostic efficiency of hospital microbiologists, the dispensing habits of pharmacists as well as the inappropriate use of antibiotics. Good hygienic measures are of great importance in controlling possible transmission of *Pseudomonas aeruginosa* infections within and outside the hospital environment.

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1. Introduction.

Multidrug resistance (MDR) *P. aeruginosa* has been one of the major challenges faced by medical personnel and has caused significant hospital-associated outbreaks of infection (Bukholm et al., 2002; Pena et al., 2003). Vehicles of disease transmission implicated in such outbreaks include antiseptic solutions and lotions; endoscopy equipment; ventilator apparatus; and mouth swabs (Engelhart et al., 2002; Silva et al., 2003). Sometimes however, sources of an outbreak may be traceable to hospital infrastructures, such as plumbing fixtures.

Susy et al. (2009) identified an outbreak strain of *P. aeruginosa* resistant to all anti-pseudomonal antibiotics such as ceftazidime, imipenem, ciprofloxacin, piperacillin-tazobactam, and gentamicin. In a recent study, Isibor et al. (2013) found a high percentage of multidrug resistant strain of *P. aeruginosa* associated with diabetic wounds of patients. According to Carmeli et al., 2002 the risk for acquiring multidrug resistant organisms is most likely to be related to the number of carriers in the same ward as well as to individual risk factors, such as patient characteristics and in-hospital events (invasive devices and antibiotic treatment.

In this study, *P. aeruginosa* was isolated from clinical specimens and their antibiotic susceptibility pattern and plasmid profile of the multidrug resistance strains were determined.

2. Materials and methods

Collection of specimens

A total of 101 clinical specimens (40 urine and 61 swab specimens), were randomly collected from patients attending some clinics in Benin City. Urine specimens were collected in sterile universal bottles, while swab specimens were collected using sterile swab sticks.

Ethical clearance

Approval was obtained from the Medical Directors of the hospitals whose patients participated in this study and the patients gave their consent after being informed of the objectives of study.

Bacteriological procedures

Specimens were aseptically inoculated onto MacConkey, Blood and Nutrient agar and incubated aerobically at 37^{o}C for 24 hours and observed for colonial growth.

Identification of isolates

Isolates were identified by their colonial morphology, Gram reaction, motility, oxidase positivity, pigment production and growth at 42^{o}C (Cheesbrough, 2000).

Antibiotic susceptibility testing

Susceptibility to antibiotics was assessed using the Kirby-Bauer disc diffusion method, and zones of inhibition were read using the Clinical and Laboratory Standards Institute's guidelines (CLSI, 2010).

Plasmid Curing Procedure

Multidrug resistance isolates were subjected to plasmid curing experiment using the modifications of Olukoya and Oni (1990). Overnight cultures in nutrient broth were diluted 10-fold and 1ml inocula were added to 30ml of nutrient broth (pH; 7.6).Then 1ml of 10%^{w}/v sodium dedocyl sulfate (SDS) solution was added to the broth and incubated for 24 hours. The overnight broth cultures were diluted with sterile distilled water and inoculated onto Mueller Hinton agar plates. The colonies were then sub-cultured onto Mueller Hinton agar (Difco Laboratories, Detroit, Mich) plates and were again screened for antibiotic resistance by the disk diffusion method, following the Clinical and Laboratory Standards Institute's guidelines. Resistance markers expressed after curing were regarded as being chromosome-mediated while those not expressed were regarded as plasmid mediated.

Plasmid DNA Extraction and Gel electrophoresis

Plasmid extraction was carried out using the method described by Birnboim and Doly (1979). Isolated plasmids were thereafter electrophoresed in a horizontal tank at a constant voltage of 90V for 60 minutes. After electrophoresis, plasmid DNA bands were viewed under UV transillumination and photographed using a digital camera. The DNA bands were compared with those for the lambda DNA *Hind*Ill digest molecular weight marker (Promega Corporation) which ranged in size from 100bp to 1000bp, and results recorded.

3. Results

In this study, 32 (32%) of the 101 specimens yielded *Pseudomonas aeruginosa*. Table 1 shows the antibiotic susceptibility pattern derived from this study. The resistance rate was in the following decreasing order: Nalidixic acid and Ceporex (92.3%), Ampicillin (76.9%), Augmentin and Gentamicin (46.2%), Septrin (38.5%), Ciprofloxacin and Reflacine (30.8%), Streptomycin (15.4%) and Ofloxacin (7.7%). Multi-drug resistance phenotype was found in 13(40.6%) of the isolates (Table 2). One isolate was resistant to 8 antibiotics tested.

The number and percentage of isolates showing resistance, before and after plasmid curing, and the percentage of isolates cured of their plasmids are shown in Table 3.Table 4 shows the frequency of multiple antibiotic resistance (MAR). MAR was determined using the formula MAR=x/y, where x is the number of antibiotics to which test isolate displayed resistance and y is the total number of antibiotics to which the test organism has been evaluated for susceptibility (Akinjogunla and Enabulele, 2010).

The agarose gel electrophoresis profiles of plasmid DNA of clinical isolates of *P. aeruginosa* are indicated in Figures 1 and 2. Isolates 2, 6, 7, 9 and 10 show plasmid bands at 300bp. Isolates 1, 4, 5, 8 and 11band at 150bp while lanes 3 and 12 are negative for plasmid genes.

Table 1. Antibiotic susceptibility pattern of *P. aeruginosa*

Antibiotics	Concentration (µ)	No (%) of Resistant Isolates	No (%) of Sensitive Isolates
Ampicillin	(30)	10(76.9)	3(23.1
Augmentin	(30)	6(46.2)	7(53.8)
Streptomycin	(30)	2(15.4)	11(84.6)
Gentamicin	(10)	6(46.2)	7(53.8)
Ceporex	(10)	12(92.3)	1(7.7)
Septrin	(30)	5(38.5)	8(61.5)
Ofloxacin	(10)	1(7.7)	12(92.3)
Ciprofloxacin	(10)	4(30.8)	9(69.2)
Nalidixic acid	(30)	12(92.3)	1(7.7)
Reflacine	(10)	4(30.8)	9(69.2)

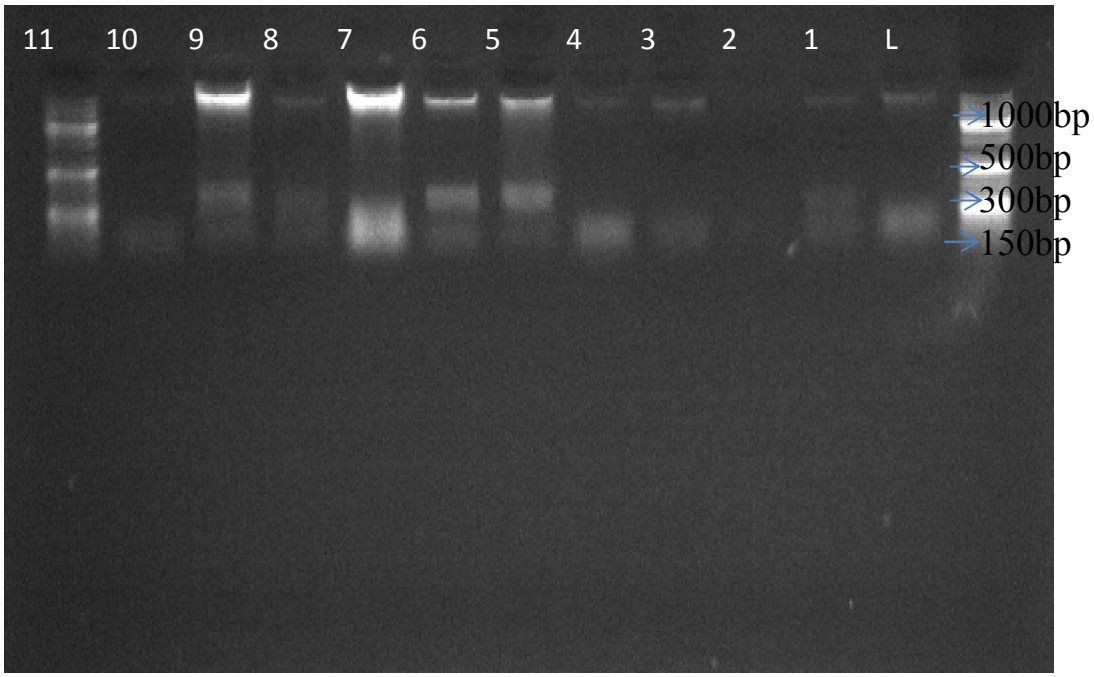

Figure 1. Agarose gel electrophoresis showing profiles of plasmid DNA of clinical isolates of *P. aeruginosa*. Lane L is the molecular weight marker (λ DNA/*Hind* III digest) of size range 100bp to 1000bp. Isolates 2, 6, 7, 9 and 10 show plasmid bands at 300bp, while isolates 1, 4, 5, 8 and 11band at 150bp. Lane 3 is negative for plasmid gene.

Table 2. Summary of Antibiotic Resistance Profile of *P. aeruginosa* (N=13)

Number of antibiotics to which there was resistance	Number (%) of strains showing resistance pattern
Three antibiotics	2(15.4)
Four antibiotics	6(46.2)
Five antibiotics	1(7.7)
Six antibiotics	2(15.4)
Seven antibiotics	1(7.7)
Eight antibiotics	1(7.7)
Number (%) of MDR strains	13(40.6)

Table 3. Plasmid curing analysis of *P. aeruginosa isolates*. (N=13)

Antibiotics	No (%) of resistant (µg) curing	No (%) of Isolates cured isolates pre-curing	No (%) of isolates post-	resistant of their plasmids
Ampicillin	(30)	10(76.9)	1(7.7)	9(90)
Augmentin	(30)	6(46.2)	1(7.7)	5(83)
Streptomycin	(30)	2(15.4)	0(0.0)	2(100)
Gentamicin	(10)	6(46.2)	2(15.4)	4(67)
Ceporex	(10)	12(92.3)	3(23.1)	9(75)
Septrin	(30)	5(38.5)	1(7.7)	4(80)
Ofloxacin	(10)	1(7.7)	1(7.7)	0(00)
Ciprofloxacin	(10)	4(30.8)	1(7.7)	3(75)
Nalidixic acid	(30)	12(92.3)	1(7.7)	11(92)
Reflacine	(10)	4(30.8)	1(7.7)	3(75)

Table 4. Frequency of multiple antibiotic resistance (MAR*) and multiple antibiotic resistance indices of *P. aeruginosa* isolates.

No. of antimicrobial agents to which isolates are resistant.	No. of isolates with MAR.	MAR indices.
3 antibiotics	2	0.3
4 antibiotics	6	0.4
5 antibiotics	1	0.5
6 antibiotics	2	0.6
7 antibiotics	1	0.7
8 antibiotics	1	0.8

* MAR index = No. of antimicrobial agents isolate is resistant to
No. of antimicrobial agents tested (that is, 10).

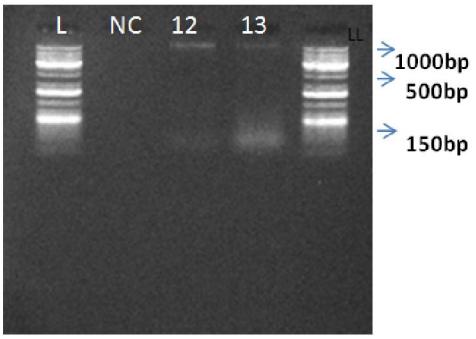

Figure 2. Agarose gel electrophoresis showing profiles of plasmid DNA of clinical isolates of *P. aeruginosa*. Lane L is the molecular weight marker (λ DNA/*Hind* III digest) of size range 100bp to 1000bp. Lane 12 is negative for plasmid gene, while Lane 13 is positive for plasmid gene at 150bp. NC is Negative control.

4. Discussions

In this study, an overall prevalence rate of Pseudomonas isolation was 32%. The preponderance of this pathogen in nosocomial infections is no longer in doubt. In Pakistan, Saghir et al. (2009) and Zaman et al. (2013) found isolation rates of 38% and 33% in hospital patients respectively. Of the six different species of bacteria isolated from hospital patients in Tamil Nadu, India, *P. aeruginosa* had the highest prevalence rate of 43 % (Manikandan and Amsath, 2013).

Out of the 32 *P. aeruginosa* isolates in this study, 13(40.6%) were multi-drug resistant. Multidrug resistance *P. aeruginosa* have previously been reported among patients with diabetic wounds in Irrua, Nigeria (Isibor et al. 2013). In an Iranian study (Salami et al. 2009) multidrug-resistance *P. aeruginosa* had a prevalence rate of 42 (33%) out of 127 clinical isolates investigated. Smith et al. (2010) however recorded a high 60.0% prevalence for multidrug resistance *P. aeruginosa* isolated from surgical wounds in Lagos, Nigeria.

Multidrug-resistance *P. aeruginosa* is a public health concern that affects many countries of the world. The various virulence mechanisms possessed by this pathogen as well as patients' risk factors such as immunosuppresion, age, likely drug interactions, antibiotic misuse, long hospital stay, may contribute to the varying resistance rates. According to Carmeli et al. (2002) the risk for acquiring multidrug resistant organisms is most likely related to the number of carriers in the same ward as well as to individual risk factors.

Among the quinolones tested in this study, *P. aeruginosa* had in-vitro sensitivity of 92.3% to Ofloxacin, followed by Ciprofloxacin (69.2%), and Reflacine (69.2%) (Table1). A sensitivity rate of 73.6% has also been documented for Ciprofloxacin (Zahra and Moniri, 2011). Contrary to these high rates, lower sensitivity rates (18.2% and 43.6%) to Ciprofloxacin have been reported by Gad et al. (2007) and Mahmoud et al. (2013) respectively. These observed differences in the response of the organism to Ciprofloxacin may result from differences in patient's individual immunity and previous exposure to related types of antibiotic.

The resistance rate to Nalidixic acid was 92.3%, which compares favorably with the study carried out in Pakistan (92%) by Zaman et al., 2013. A 100% resistance rate of *P. aeruginosa* to Nalidixic acid was also recorded in Nigeria (Smith et al., 2010; Anthony et al., 2010). The high resistance rates may have been caused by spontaneous mutations in bacterial cells during treatment, thus making the target site inaccessible to antibiotic action, thereby resulting to increased resistance to the selective action of Nalidixic acid. Although fluoroquinolones, according to Gasink et al. (2006), were the only oral therapy available for *P. aeruginosa* infections, fluoroquinolone-resistant *P. aeruginosa* has increased significantly and this could be associated with prior fluoroquinolone use by patients.

Table 4 shows the frequency of Multiple Antibiotic Resistance (MAR), defined here as joint resistance shown by isolates to more than two antibiotics (Ngwai et al., 2011; Ajayi et al., 2011). It can be seen that MAR was present in all the isolates, with the MAR indices ranging from 0.1 to 0.4. One isolate was at the same time resistant to 8 of the 10 antibiotics tested. According to Krumperman (1983), MAR indices above 0.2 indicate that such isolates originate from an environment where antimicrobial

agents are freely available and accessible with high potential for abuse.

The fact that our study specimens were also obtained from hospital patients clearly justifies this claim. It has been suggested that cross-carriage or colonization/infection seems to play an important role in the general spread of *P. aeruginosa* in the hospital intensive care unit (Bertrand et al., 2001). Li et al. (2000) have suggested that the synergy between outer membrane impermeability and chromosomally-encoded multidrug efflux pumps could result to the intrinsic multidrug resistance of this organism. Nevertheless, the species inherent resistance to various antibiotics is also largely dependent on the acquisition and transfer of resistant plasmids.

In this study, eleven (84.6%) of the resistant isolates harbored plasmids with bands ranging from 150bp to 300bp (Fig. 1and 2). Resistance to antibiotics has been ascribed in most instances to the presence of plasmids (Daini et al., 2006). In the study carried out in Benin City, Nigeria, 11.4% of the Pseudomonas isolates was plasmid-mediated, and were highly transferable with a frequency range of $2x10^{-2}$ to $6x10^{-4}$ (Yah et al., 2006).

Concerted strategies at monitoring prescribing habits of clinicians, the diagnostic efficiency of hospital microbiologists, the dispensing habits of pharmacists, the inappropriate use of antibiotics, as well as encouraging good hygienic measures could help curtail possible transmission of MDR *P. aeruginosa* infections within and outside the hospital environment.

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