

Plasmid Profiling and Curing Analysis of Fluoroquinolone Multidrug Resistant *Pseudomonas aeruginosa* in Benin City, Nigeria

F. A. Ehiaghe^{1,2,3*}, I. J. Ehiaghe^{2,3}, D. E. Agbonlahor^{2,4}, F. E. Oviasogie⁵,
S. M. O. Etikerentse¹, R. A. U. Nwobu³, B. O. Akinshipe⁶, A. I. Ikusemore¹,
S. T. Aladenika⁶, F. O. Enwa⁷

¹Department of Hematology, College of Health Sciences, Igbinedion University, Okada, Nigeria

²Lahor Research and Medical Centre, 121 Old Benin—Agbor Road, Benin City, Nigeria

³Department of Medical Laboratory Science, Nnamdi Azikiwe University, Awka, Nigeria

⁴Department of Medical Laboratory Science, College of Health Sciences Niger Delta University, Wilberforce Island, Amassoma, Bayelsa State

⁵Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria

⁶Department of Medical Microbiology, College of Health Sciences, Igbinedion University Okada, Okada, Nigeria

⁷Faculty of Pharmacy, Delta State University, Abraka, Nigeria

Email: *fredleo2547@yahoo.com

Received April 27, 2013; revised May 27, 2013; accepted June 4, 2013

Copyright © 2013 F. A. Ehiaghe *et al.* This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

ABSTRACT

Aim: To determine the Plasmid DNA profile of the multidrug resistant strains of *Pseudomonas aeruginosa* in the clinical isolates. **Materials and Methods:** Of the 150 clinical samples (Ear swab, Urine, Wound swab, Sputa and Semen) received at Lahor Research Laboratory and Medical center in Benin City, between January 2010 and December 2012, 36 (24%) yielded significant growth of *P. aeruginosa*. Samples were cultured on MacConkey and Blood agar. Clinical isolates were identified using standard method. Antibiotics susceptibility test employing agar disc diffusion method was used. Clinical isolates were subjected to Plasmid DNA profiling and curing test was carried out at Lahor Molecular Laboratory. This was followed by a post plasmid curing susceptibility test. Agarose gel electrophoresis was carried out to separate the Plasmid DNA using standard method. Bands were visualized using UV illuminator. **Results:** Wound swabs had the highest numbers of clinical isolates of *P. aeruginosa* (55.6%) followed by Urine, Semen, Sputa and Ear swab (19.4%, 11.0%, 8.3%, and 5.6%) respectively. Before the isolates were cured of their plasmid, 39% of the *P. aeruginosa* strains were found to be resistant to Ciprofloxacin (CPX), 47%, Ofloxacin (OFX), 44% Pefloxacin (PEF) and 56% Sparfloxacin (SPX). After plasmid curing, the new antibiogram of the isolates showed that some clinical isolates that hitherto were resistant to a given Fluoroquinolone became susceptible, 36% to CPX, 12% to OFX, 12.5% to PEF and 15% to SPX. Agarose gel electrophoresis carried out on the Plasmid DNA revealed that there was detectable Plasmid DNA in 13.9% of the clinical isolates analyzed. **Conclusion:** There is an alarming increase of clinical infections caused by multidrug resistant strains of *P. aeruginosa*. 13.9% of the multidrug resistance strains of *P. aeruginosa* in Benin City were Plasmid mediated. Treatment should be based on current Laboratory Susceptibility Test results of the isolates.

Keywords: *Pseudomonas aeruginosa*; Fluoroquinolone; Plasmids Analysis; Benin City

1. Introduction

Pseudomonas aeruginosa is a Gram-negative aerobic, coccobacillus bacterium with unipolar motility. In mixed cultures, it can be isolated as clear colonies on MacConkey agar (as it does not ferment lactose) which will test

positive for oxidase [1]. It is often preliminarily identified by its pearlescent appearance and grape like odor invitro. Definitive clinical identification of *P. aeruginosa* often includes identifying the production of pyocyanin and fluorescein as well as its ability to grow at 42°C. *P. aeruginosa* is capable of growth in diesel and jet fuel, where it is known as a hydrocarbon-using microorganism

*Corresponding author.

[2].

The genome of *P. aeruginosa* is relatively large (6 - 7 mb) and encodes around 6000 open reading frames (ORFs), depending on the strain. There are 5021 genes that are conserved across all 5 (five) genomes analyzed with at least 70% sequence identity [3]. One of the most worrisome characteristics of *P. aeruginosa* is its antibiotic susceptibility, which is attributable to a concerted action of multidrug efflux pump with chromosomally encoded antibiotic resistance genes (*mexAB*, *mexXY*) and the low permeability of the bacterial cellular envelopes. *P. aeruginosa* can easily develop acquired resistance either by mutation in chromosomally encoded genes or by the horizontal Plasmid DNA transfer of antibiotic resistance determinants [4].

Plasmid DNA are extra chromosomal element of finite size, usually stably inherited within a bacterial cell line and potentially capable of transfer between strains, species or genera [5]. They were first discovered in enteric bacteria and from the late 1950s onwards were increasingly associated with antibiotics resistance. Its main function is to carry antibiotics resistant genes, which are responsible for the increase pathogenicity of most bacteria [5,6].

The aim of this investigation is to determine the antimicrobial susceptibility of clinical isolates *P. aeruginosa* to four Fluoroquinolone and to determine the percentage of multidrug resistance of *P. aeruginosa* strains that are plasmid mediated.

2. Materials and Methods

2.1. Samples Collection

150 samples were collected from Lahor Research Laboratory and medical center, 121 Old Benin-Agbor Road, Ikpoba Hill, Benin City, Edo state, Nigeria. Of which include specimen of ear swab, wound swab, mid stream urine, sputum and semen. Samples were collected between January 2010 to December 2012. Ethical approval and patient consent was obtained before sample collection.

2.2. Cultural Methods

With a grease pencil, the bottom of MacConkey agar and Blood agar Plates were divided into three parts; the samples were cultured on the media and incubated at 37°C for 24 to 48 hours depending on the nature of the sample. Of the 150 samples collected, 36(24%) yielded significant growth of *P. aeruginosa* and were stored on nutrient agar slant of MarCartney bottle.

2.3. Identification of Isolates

The isolates were identified by standard techniques [7]. This included colonial appearance of the organism on the

media, morphological characteristics such as size, form, elevation, opacity, colour, odour and edge. Gram's technique was used to classify the organism into Gram negative coccobacillus; they were further subjected to specific biochemical tests such as citrate utilization, indole production, urease production, oxidase test, sugar fermentation (sucrose, salicin, lactose, maltose and mannitol) test along side with motility test.

2.4. Antibiotics Susceptibility Testing

Susceptibility tests were performed on each *P. aeruginosa* isolated using the agar disc diffusion method described by [8]. Antibiotic disc of Ciprofloxacin (10 ug), Ofloxacin (10 ug), Pefloxacin (10 ug) and Sparfloxacin (10 ug) were used. Zones of inhibition less than 13 mm were regarded as resistant while those measuring 15 mm and above as susceptible. The multi resistant isolate that were resistant to all the four Antibiotics were taking for Plasmid DNA curing.

2.5. Plasmids Curing

Isolates that were resistant to the Fluoroquinolone were subjected to standard plasmid curing method [9].

- Overnight broth culture was inoculated into 4.5 ml nutrient broth.
- 0.5 ml of Sodium deodecyl sulphate was added and Incubated for 48 hours at 37°C.
- 0.5 ml of the broth was added into a freshly prepared 4.5 ml nutrient broth, it was incubate for another 24 hours at 37°C.
- After which post plasmid Antibiotic susceptibility was carried out.

On each of the clinical isolates of *P.aeruginosa* as described [8].

The reason for this second susceptibility test is to check if the curing agent has removed the resident plasmid present in the resistant isolates.

Preparations of Reagents

Reagents	Composition Reagents
1. Buffer 1A (Cell Resuspension Buffer)	50 mM Tris-Cl, pH 8.0 10 mM EDTA 100 ug/ml DNase free RNase
2. Buffer 2B (Neutralization solution)	3.0 mM Potassium acetate, pH 5
3. Buffer 3C (Equilibration Buffer)	6 M guanidine hydrochloride
4. Cell Lysis Buffer	200 mM NaOH 1% Sodium deodecyl sulphate 0.8% Agarose Gel

5. 100 ml of Tris Boric EDTA (TBE Buffer)	preparation
	108 g Tris
	55 g Boric acid
	40 ml of 0.5 M EDTA, pH 8
	8 g of Agarose powder

2.6. Agarose Gel Electrophoresis

Plasmid isolation was performed using the alkaline lysis method described by [10]. After isolation of plasmid DNA, a horizontal agarose gel electrophoresis was carried out with the method described by [11].

The plasmid profiling was carried out by sub-culturing those isolate onto nutrient agar plate and incubated for 24 hour at 37°C. The isolate was harvested into Eppendorf tube. 200 µl of buffer 1A (Cell Resuspension Buffer) was added and vortexed for proper mixing. 200 µl of Cell lysing solution was added. The tubes were Inverted 20 times at room temperature. Add 300 µl of ice-cold buffer 2B (Neutralization solution), vortexed and keep on ice for 30 minutes, centrifuged at 3000 × g for 15 minutes. To supernatant, 700 µl of chloroform was added and centrifuged at 3000 × g for 10 minutes. To 500 µl aqueous layer, 1 ml of absolute ethanol was added and kept in ice for 1 hour. This was centrifuged at 3000 × g for 30 mins and the pellet was washed in 70% ethanol. 100 µl of buffer 3C (Equilibration Buffer) was added to the dried pellet.

After the DNA have been extracted from the cells, 0.8% agarose gel with wells was prepared, in which 2 ul of the loading dye was mixed with 10 ul of the 100 base pair molecular markers and transferred into the first well. 2 ul of the loading dye was mixed with the sample and it was transferred into the other wells. Agarose gel Electrophoresis was carried out on the plasmid DNA at 90 v for 60 minutes. It was viewed under UV Trans-illumination for the band harboring plasmid.

3. Results

The results obtained are shown in **Tables 1-3** and **Plate 1**.

Table1: Shows *P. aeruginosa* from various clinical sources. Isolates were highest from wound swabs.

Table 2: Shows the Antibioqram pre Plasmid curing of *P. aeruginosa* isolates. Whereas isolates showed the highest resistance to Sparfloxacin (56%), they exhibited the lowest resistance to Ciprofloxacin (39%).

Table 3: Shows the Antibioqram post Plasmid Curing of multidrug resistant strains of *P. aeruginosa* to four Fluoroquinolone. Of the 20 strains that were resistant to Sparfloxacin, 3 were plasmid mediated while none was plasmid mediated in the 14 strains that were resistant to Ciprofloxacin.

Plate 1: Shows the Results of the Plasmids DNA profiling analyzed in a 0.8% Agarose gel Electrophoresis. Five samples 16, 18, 26, 28 and 29 were plasmid positive.

4. Discussion

Our study revealed that wound swab had the highest number of clinical isolates of *P. aeruginosa* (55.6%) followed by urine sample (19.4%), Semen (11%) and Sputa sample (8.3%). These may be due to the fact that surgical wound sample site are exposed to many ubiquitous environmental pathogen which include *P. aureginosa* found in unsterile surface, water and soil. These findings have been reported by [12]. *P. aeruginosa* has a prevalence of 29.6% among pathogens isolated from surgical wound site. The infection appears to be common in hospital with relaxed hygienic measure. This is also in accordance with these findings. It is the most common pathogen causing wound infection especially in developing countries. It is an epitome of opportunistic nosocomial pathogen, which causes a wide spectrum of infection and lead to substantial morbidity in immune compromised patients [13-15].

It was observed that at Antibioqram pre curing stage of *P. aeruginosa*, the results show that 39% of the microorganism was found to be resistant to Ciprofloxacin, 47% to Ofloxacin, 44% to Pefloxacin and 59% to Sparfloxacin. This finding has been reported by other Authors. A high number of acquired resistance genes coding for aminoglycoside modifying enzymes and β -Lactamases have been noted in *P. aeruginosa* [16]. Extended spectrum

Table 1. *P. aeruginosa* isolated from various sources.

Source/site	Number of isolate (%)	Biochemical reaction						
		Citrate	Oxidase	Urea	Lactose	Motility	Growth 42°C	Gram Reaction
Ear swab	2 (5.6)	+	+	+	-	+	+	-
Urine	7 (19.4)	+	+	+	-	+	+	-
Wound swab	20 (55.6)	+	+	+	-	+	+	-
Sputum	3 (8.3)	+	+	+	-	+	+	-
Semen	4 (11)	+	+	+	-	+	+	-
Total	36							

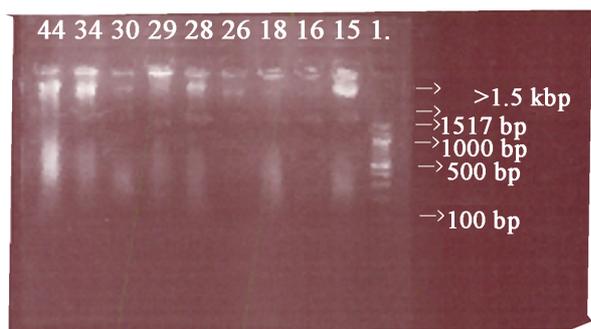
Table 2. Antibiogram pre curing of *P. aeruginosa* to four fluoroquinolone.

Population	CPX	OFX	PEF	SPX
Susceptible	22 (61.1%)	19 (52.8%)	20 (56.0%)	16 (44.4%)
Resistant	14 (39.0%)	17 (47.0%)	16 (44.4%)	20 (56.0%)

Keys: 1. CPX—Ciprofloxacin; 2. OFX—Ofloxacin; 3. PEF—Pefloxacin; 4. SPX—Sparfloxacin.

Table 3. Antibiogram post plasmid curing of multidrug resistant strains of *Pseudomonas aeruginosa* to four fluoroquinolone.

Population	CPX	OFX	PEF	SPX
Susceptible	0 (0%)	1 (5.9%)	1 (6.0%)	3 (15%)
Resistant	14 (100%)	16 (94.1%)	15 (94%)	17 (85%)

**Plate 1. Results of the plasmid DNA profile analysed in a 0.8% agarose gel, L is a DNA ladder with size range of 0.1 - 5.1 kbp, samples 16, 18, 26, 28 and 29 have a plasmid band greater than 1.5 kbp.**

β -Lactamases have been increasingly reported about this micro organism [17]. Metallo β -Lactamases have also emerged in *P. aeruginosa* [18].

Once colonization and infection are established, *P. aeruginosa* becomes one of the worst pathogens of human and it is known to possess intrinsic multi-drug resistance capabilities [19,20]. It has a large genome of about 6.3 million base pairs (bp), house 8 virulence genes, the large genome size increase the probability of possible mutation sites and thus gives reasons for its virulence versatile, its growing multi-drug resistance and the high mortality rate associated with its infection [21].

It was also observed that at Antibiogram pre-curing stage of *P. aeruginosa*, results revealed that 61.1% of the isolates were sensitive to CPX, 52.8% to OFX, 56.0% to PEF and 44.4% Sparfloxacin. It might be due to that some clinical isolates of *P. aeruginosa* do not hyper expressed the efflux system genes that confer resistance to Antibiotics. This is in line with these finding. The hyper expression of MexAB-OprM efflux pump mainly found in *P. aeruginosa* are not present in some clinical isolates of *P. aeruginosa*, hence they are susceptible to most Antibiotics [22].

At Antibiogram post curing stage of *P. aeruginosa*, our result shows that some clinical isolates that were resistant to the same Fluoroquinolone at Antibiogram pre-curing stage became susceptible at the Antibiogram post curing stage. 5.9% to OFX, 6.8% to PEF and 15% to SPX. These might be due to the fact that the plasmid borne multidrug resistance gene had been denatured or removed by the sodium deodecyl sulphate used as the curing agent. This is in agreement with these findings. *P. aeruginosa* is a classic opportunistic pathogen especially because of its innate resistance to many antibiotics and its acquired resistance due to plasmid mediation [23].

The plasmids analysis revealed that there were detectable plasmid DNA genes in sample 16, 18, 26, 28 and 29, which represents 13.9% of the multidrug resistance strains of the clinical isolates analyzed. The plasmids DNA genes isolated in the 0.8% Agarose gel Electrophoresis might be the bacterial contents that confer plasmid resistance on the clinical isolates of *P. aeruginosa*. This is in accordance with these findings. The plasmids profile analysis examines the total bacterial plasmid content and is separated by electrophoresis. This method is used to assess the spread of antibiotics plasmid DNA resistance determinates [23].

5. Conclusion and Recommendation

There is an alarming increase of clinical infection caused by multidrug resistant strains of *P. aeruginosa*. Plasmid analysis revealed that 13.9% of the multidrug resistant strains of *P. aeruginosa* were plasmid mediated in Benin City. Treatment should be based on current laboratory susceptibility test results.

REFERENCES

- [1] K. J. Ryan and C. G. Ray, "Sheris Medical Microbiology," 4th Edition, McGraw Hill, New York, 2004.
- [2] A. Balcht and R. Smith, "*Pseudomonas aeruginosa*: Infection and Treatment," *Informa Healthcare*, 1994, pp. 83-84.
- [3] K. Mathee, G. Narasimhan, C. Valdes, X. Qiu, J. M. Metewish, M. Koehrsen, A. Rokas and C. N. Yandava, "Dynamics of *Pseudomonas aeruginosa* Genome Evolution," *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 105, No. 8, 2008, pp. 3100-3105. doi:10.1073/pnas.0711982105
- [4] K. Poole, "Efflux Mediated Multiresistance in Gram-Negative Bacteria," *Clinical Microbiology and Infection*, Vol. 10, No. 1, 2004, pp. 12-26. doi:10.1111/j.1469-0691.2004.00763.x
- [5] V. Alan, M. Jesus, and W. J. Rober, "The Role of Plasmid in Phytopathogenic Bacteria: Mobile Arsenals," *Journal of General Virology*, Vol. 147, No. 4, 2001, pp. 4763-4780.
- [6] N. Datta and V. M. Hughes, "Plasmid of the Same Inc.

- Group in Enterobacteria before and after the Medical Use of Antibiotics,” *Nature*, Vol. 306, No. 5943, 1983, pp. 616-613. [doi:10.1038/306616a0](https://doi.org/10.1038/306616a0)
- [7] S. T. Cowan and S. Steel, “Manual for the Identification of Medical Bacteria,” 3rd Edition, Cambridge University Press, Cambridge, 1974.
- [8] Clinical Laboratory Standard Institute, “Performance Standard for Antimicrobial Disk Susceptibility Tested: Approved Standard,” 2006.
- [9] T. J. Sijhary, M. L. Bermann and L. W. Enquist, “Experiments with Gene Fusions,” Cold Spring Harbor Laboratory Press, New York, 1984.
- [10] H. Bimboim and J. Doly, “A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA,” *Nucleic Acids Research*, Vol. 7, No. 6, 1979, pp. 1513-1523. [doi:10.1093/nar/7.6.1513](https://doi.org/10.1093/nar/7.6.1513)
- [11] J. A. Meyers, D. Sanchez, L. P. Elwell and S. Falkow, “Simple Agarose Gel Electrophoresis Method for the Identification and Characterization of Plasmid Deoxyribonucleic Acid,” *Journal of Bacteriology*, Vol. 127, No. 3, 1976, pp. 1529-1537.
- [12] K. P. Ranjan, N. Ranjan, K. S. Bansal and P. R. Arora, “Prevalence of *P. aeruginosa* in Most Operative Wound Infection in a Referral Hospital in Haryana,” *Indian Journal of Laboratory Physicians*, Vol. 2, No. 2, 2010, pp. 74-77. [doi:10.4103/0974-2727.72153](https://doi.org/10.4103/0974-2727.72153)
- [13] N. M. R. Bojary and M. Hajia, “Multidrug Resistant *P. aeruginosa* Strains in Tehran Reference Burn Hospital, Tehran, Iran,” *African Journal of Microbiology Research*, Vol. 6, No. 7, 2012, pp. 1393-1396.
- [14] M. Hajia, M. Qorbanalizadehgan, M. Rahbar and M. Izadi, “Laboratory Evaluations of Iranian Commercially Provided Antibiotic Disks with Conventional E-Test for Susceptibility Testing in Three Most Isolated Multi-Drug Resistant Organisms,” *Internet Journal of Microbiology*, Vol. 5, 2008, pp. 23-28.
- [15] M. Rahbar, H. Mehragan and N. Hajiali-Akbori, “Prevalence of Drug Resistance in Non fermenter Gram Negative Bacilli,” *Iranian Journal of Pathology*, Vol. 5, 2010, pp. 90-96.
- [16] R. A. Bonomo and D. Szabo, “Mechanisms of Multidrug Resistance in Acinetobacter Species and *Pseudomonas aeruginosa*,” *Clinical Infectious Diseases*, Vol. 43, No. 2, 2006, pp. 49-56. [doi:10.1086/504477](https://doi.org/10.1086/504477)
- [17] G. F. Weldhagen, L. Poirel and P. Nordmann, “Ambler Class A Extended Spectrum β -Lactamases in *Pseudomonas aeruginosa*: novel developments and Clinical Impact,” *Antimicrobial Agents and Chemotherapy*, Vol. 47, No. 8, 2003, pp. 2385-2392. [doi:10.1128/AAC.47.8.2385-2392.2003](https://doi.org/10.1128/AAC.47.8.2385-2392.2003)
- [18] T. R. Walsh, M. A. Toleman, L. Poirel and P. Nordmann, “Metallo- β -Lactamases: The Quiet before Storm? *Clinical Microbiology Reviews*, Vol. 18, No. 2, 2005, pp. 306-325. [doi:10.1128/CMR.18.2.306-325.2005](https://doi.org/10.1128/CMR.18.2.306-325.2005)
- [19] G. Agarwal, A. Kapil, S. K. Kabra, B. K. Das and N. Dwivedi, “Characterization of *Pseudomonas Aeruginosa* Isolated from Chronically Infected Children with Cystic Fibrosis in Indian,” *BMC Microbiology*, Vol. 5, No. 1, 2005, pp. 43-47. [doi:10.1186/1471-2180-5-43](https://doi.org/10.1186/1471-2180-5-43)
- [20] H. Y. Chen, K. Yunn and D. M. LiverMore, “Mechanism of Resistance to Beta-Lactam Antibiotics among *Pseudomonas Aeruginosa* Isolates Collected,” *Medical Microbiology*, Vol. 43, No. 4, 1995, pp. 300-309.
- [21] S. Rahmatis, S. Yang, A. L. Davidson and E. L. Zechiedrich, “Control of the Acr AB Multi-Drug Efflux Pump by Quorum Sensing Regulator,” *Molecular Microbiology*, Vol. 43, No. 3, 2002, pp. 677-850. [doi:10.1046/j.1365-2958.2002.02773.x](https://doi.org/10.1046/j.1365-2958.2002.02773.x)
- [22] M. Shadid and N. Malik, “Plasmid Mediated Amikacin Resistance in Clinical Isolate of *Pseudomonas aeruginosa*,” *Indian Journal of Medical Microbiology*, Vol. 22, No. 3, 2004, pp. 182-184.
- [23] R. C. Clowers, “Molecular Structure of Bacterial Plasmids,” *Bacteriological Reviews*, Vol. 36, No. 3, 1972, pp. 361-405.