EFFLUX MEDIATED MULTIDRUG RESISTANT *PSEUDOMONAS AERUGINOSA* ISOLATED FROM DIFFERENT ENVIRONMENTAL SOURCES

*1G.C. AGU, 1B.T. THOMAS, 2O.O. SALAMI AND 1O.D. POPOOLA

1Department of Microbiology, Olabisi Onabanjo University, Ago-Iwoye, Nigeria
2Science Laboratory Technology Department (Microbiology Unit), Federal College of Animal Health and Production Technology, Moor Plantation, Apata Ibadan, Nigeria
***Corresponding Author**:georgiaagu@yahoo.com   Tel: +2348034044101

ABSTRACT

Pseudomonas aeruginosa is an important opportunistic pathogen and one of the leading causes of multi-drug resistant nosocomial infections. This study was therefore carried out to determine the resistance nature, and the role of efflux pump in multidrug resistance of *Pseudomonas aeruginosa* isolated from different environmental sources using the efflux pump inhibitor, Carbonyl Cyanide 3-Chlorophenylhydrazone (CCCP). A total of 220 environmental samples were collected and processed following standard techniques. Susceptibility to antibiotics was performed using disc diffusion methods as described by the Clinical and Laboratory Standards Institute. Activity of the efflux pump system was carried out using the efflux pump inhibitor, CCCP. Results obtained identified 100 (45.5%) *Pseudomonas aeruginosa* and 72 (32.7%) other strains of *Pseudomonas* spp. The susceptibility testing revealed that all the identified strains of *Pseudomonas aeruginosa* that were subjected to susceptibility test were significantly resistant to ampicillin and cefotaxime, But the resistance profile of isolates to tetracycline, chloramphenicol, ceftriaxone, cefuroxime and perfloxacin were 93%, 72.1%, 79.1%, 58.1% and 51.2% respectively. However, imipenem was the most sensitive (100%), followed by cefepime (65%) and gentamicin (44%). Carbonyl Cyanide 3-Chlorophenylhydrazone decreased the minimum inhibitory concentration (MIC) of the isolates by 2 folds. Results obtained have shown the ubiquitous presence of multi-drug resistant *P. aeruginosa* from the environmental samples examined. Furthermore, it indicated the role of efflux pump in antibiotics resistance in *P. aeruginosa* isolates which indicate that *P. aeruginosa strains* from environmental sources could resist antibiotics by the efflux mechanism.

Keywords: Multi-Drug Resistance, Efflux pump Inhibitor; CCCP, *Pseudomonas aeruginosa*, Environmental sources

INTRODUCTION

Pseudomonas aeruginosa is an aerobic, gram negative rod that can be found virtually everywhere: soil, plants/animals, hospital, reservoir of water, sinks, toilet water and showers [Blais *et al.*, 1999, Dubois *et al.*, 2001]. It is an opportunistic pathogen that initiates a variety of infections especially in immune-compromised patients. Infections caused by this organism results in significant morbidity and mortality [Brenwald *et al.*, 2000]. *Pseudomonas aeruginosa* was reported to be the sixth most common nosocomial agent and second most common pathogen in ventilator associated pneumonia in United States of American hospitals [Lawrence and Barret, 1998]. This organism is considered one of the major problems in the hospital showing

significant degree of intrinsic and acquired resistance to at least one or more antibiotics in virtually all the major classes [Beta-lactams, aminoglycosides, quinolones] of antibiotics used in the treatment of *P. aeruginosa* infections [Li *et al.*, 1994; Coats, 1998].

Gram-negative organisms, multi-drug pumps with specificity function act synergistically with other outer membrane barrier to provide intrinsic and/or acquired multi-drug resistance. It is thus well known that several strains in the family *Pseudomonadaceae* show significant resistance to a wide variety of structurally unrelated compound such as antibacterial agents and organic solvents [Kohler *et al.*,1997; Kohler *et al.*,1999a; Kohler *et al.*,1999b; Kieboom *et al.*.2000] and they are widely distributed in nature showing resistant to different antibiotics and disinfectant, in addition to its armoury of putative virulence factors plus plasmid-acquired resistance [Olayinka *et al.*, 2009].

Efflux system which presents a vital problem to chemotherapy has been defined as partial or total extrusion of antibiotics from the targeted site of the organism [Blais *et al.*, 1999; Brenwald *et al.*, 2000; Lawrence and Barrett, 1998]. This efflux system, no doubt has been broadly recognized as a major component of resistance to many classes of antibiotics. Some efflux pumps selectively extrude antibiotics, while other referred to as multi-drug resistance (MDR) pumps a variety of structurally diverse compound with differing antibacterial mode of action [Nikaido, 1996; Brown and Izundu, 2004].

In Nigeria, reports on antibiotics resistance have been established and findings on efflux mediated resistance in different foods have also been reported (Okeke *et al.*, 2000) . *Pseudomonas aeruginosa* from clinical specimens have been known for their multi-drug resistant characteristics through different mechanisms [Poirel *et al.*, 2001]. Efflux pump mediated multidrug resistant *Pseudomonas aeruginosa* of nosocomial origin have also been widely reported [Kohler *et al.*, 2001]. In Ekiti State, the antibiotic resistance pattern of *Pseudomonas aeruginosa* isolated from clinical samples had been reported by Agbalajobi *et al.* [2016] while Eyo *et al.* [2015] in Calabar reported the antibiotic resistance profiles of clinical and environmental isolates of P. *aeruginosa.* However, there is paucity of information on the multidrug resistance nature of *P. aeruginosa* isolated from environmental sources in the studied area. Considering the fact that *P. aeruginosa* is naturally resistant to many of the widely used antibiotics making treatment to be often difficult, also the fact that environment and items in the environment are prone to contamination especially in Nigeria where hygienic practices is low, standard of living is high, and antibiotic misuse and abuse is rampant. The study was therefore aimed at detecting the resistance profile, and the effect of efflux pump in multi-drug resistance of *Pseudomonas aeruginosa* isolated from the commonly used/exposed environmental items. The information or findings obtained could be helpful in devising appropriate preventive/control measures that would be of use to the society.

MATERIALS AND METHODS
Study area
This study was conducted at the Laboratory of the Department of Microbiology, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria and samples were collected from Ago-Iwoye and its environs.

Specimen collection and transportation
All the specimens were collected within one

hour of getting to the respective locations according to Blais *et al.* (1999). The soil specimens were randomly collected from different locations in agricultural areas using pre sterilized auger 8.5cm in diameter from the top 0.20cm deep into a sterilized Mac-Cartney bottles in compliance with Organization for Economics, Cooperation and Development Guideline, [2011] for soil sampling. Water samples (tap water) were aseptically collected into sterile bottles that contained 0.1% sodium thiosulphate (100mg/L) by flaming the mouth of the tap with a spirit lamp and allowed water to flow out for about 5 minutes. Sodium thiosulphate served to deactivate any chlorine that might be present in the tap water samples. Samples were also obtained from the surface of table and laboratory sink using swab stick, which were then put into nutrient broth and fully labeled, packaged and stored in an ice pack bags for microbiological analysis. The maximum time from sampling time to culture was 12 hours.

Isolation and Identification

A minimum of 30 samples were taken from different locations resulting in 220 separate samples (Table swab = 40, Lab. Sink = 30, Water sample = 30, Soil sample = 30 and Dumpsite sample = 90). Bacteriology was performed according to the standard operating procedures of the laboratory [Afiukwa *et al.*, 2011]. On receipt of the specimens, each specimen was immediately registered and processed according to the standard operating procedures of the laboratory. The samples were inoculated onto plates of solidified cetrimide agar of known sterility (Himedia, India), and incubated at 37^{0}C for 24 hours. Subcultures were carried out from 24 hours old culture to obtain pure cultures that were used for subsequent characterization tests. The plates were read for the presence of bacterial colonies. Gram staining technique was employed and the isolates were identified to species level using biochemical tests [Clinical and Laboratory Standards Institute, 2015].

Antibacterial susceptibility testing

Antibacterial assay was performed using agar disc diffusion method [Livemore, 2005; Clinical and Laboratory Standards Institute, 2015]. Mueller Hinton agar (Biotech, UK) was prepared according to the manufacturer's instruction, autoclaved and dispensed at 20 ml per plate in 12 × 12 cm Petri-dishes. The plates were incubated overnight to ensure sterility before use. Each labeled medium plate was uniformly inoculated with 0.5 McFarland standard of overnight broth culture of the test organisms using flooding method. The culture plates were dried in an incubator at 37^{0}C. A sterile forceps was used to place the different antibiotics disc on the prepared culture. All the plates after diffusion were incubated at 37^{0}C for 24 hours. Antibacterial activity was determined by measuring the diameter of zones of inhibition (mm) surrounding the bacterial growth using a transparent meter rule [Colombini *et al.*, 2000]. The result was expressed as resistant, intermediate, and susceptible, according to Clinical and Laboratory Standards Institute (2015). The experiments were repeated in duplicates and expressed as mean values of the two experiments.

Determination of the Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentration (MBCs)

The Minimum Inhibitory Concentration **(MIC)** of the antibiotics for each isolate was determined by standard dilution technique. Approximately 10^6 colony-forming unit of bacterial suspension was inoculated into nutrient broth containing varying dilutions of

the tested antibiotics to obtain a final concentration ranging from 1 - 512 µg/ml medium. Tubes were incubated at 37 ⁰C for 24 hours and the MICs were recorded as the lowest concentrations that completely inhibit the growth of the tested isolates. The minimum bactericidal concentration (MBC) was determined by pipetting, 0.1ml from each MIC concentrations as well as from the observed lower concentrations and introduce into each culture of nutrient agar plates. The plates were incubated at 37 ⁰C for 24 hours. The minimum bactericidal concentration (MBC) is the lowest concentration at which no growth occurs on solid media.

Screening of the Efflux pump Inhibitor in the resistance strains of Pseudomonas aeruginosa

The presence of efflux pump system in the resistant *Pseudomonas aeruginosa* was investigated using standard recommended methods prescribed by Radostis *et al.*[2004]; Ardebili *et al.* [2014]. Briefly, Carbonyl Cyanide 3-chlorophenyl hydrazone was incorporated to each Mueller Hinton agar plates that contained the minimum inhibitory concentration of the antibiotics. The MIC for these antibiotics was then measured again. The positive standard for the existence of efflux pump in the isolates was reduction of at least two folds or more in the MIC of the antibiotics after adding CCCP (Pumbwe *et al.,* 2006). According to Okeke *et al.,* (2000), the presence of efflux pump system is based on the reduction of the MIC value of the tested antibiotics. The MICs that further reduced after the incorporation of CCCP to the testing medium containing the *Pseudomonas* species were defined as being efflux positive.

Statistical analysis

The data obtained was subjected to statistical analysis using frequency distribution. The level of significance was set at $P < 0.05$.

RESULTS

Out of the 220 environmental samples collected, 100 (45.5%) *Pseudomonas aeruginosa* and 72 (32.7%) other strains of Pseudomonas species were identified making it a total of 172 isolates. However, 48 (21.8 %) specimens showed no sign of growth when plated (Table 1).

Table1: Prevalence of Pseudomonas species from environmental specimens

Types of specimen	Number of specimen	Other pseudomonas species (%)	*Pseudomonas aeruginosa* (%)
----------Tables	40	7(17.5)	18(45)
Laboratory sink	30	15(50)	7(23.3)
Water	30	8(26.7)	21(70)
Soil	30	13(43.3)	16(53.5)
Dumpsite	90	29(32.2)	38(42.2)
Total	220	72(32.7)	100(45.5)

Table 2 depicts the antibiotics susceptibility profiles of *Pseudomonas aeruginosa* isolates identified in the study. All the strains of *Pseudomonas aeruginosa* tested showed varied degrees of resistance except for Imipenem in which 100 % sensitivity was observed, this was followed by cefepime (65%). However, Ampicillin (100 %), and Cefotaxime, (100%) were the most resisted antibiotics. Tetracycline (93%) and Cefriazone (79.1%) were the next in the trend of resistance while gentamicin (16%) was observed to be the least resisted antibiotics by the tested isolates (Table 2).

Table 2: Antibiotic susceptibility testing of environmental *Pseudomonas*

Antibiotics	Susceptible		Intermediate		Resistant	
	No	%	No	%	No	%
Ampicillin	0.0	0.0	0.0	0.0	43	100.0
Cefuroxime	10.0	23.3	8.0	18.60	25	58.1
Ceftriazone	3.0	6.98	6.0	13.95	34	79.1
Cefotaxime	0.0	0.0	0.0	0.0	43	100.0
Cefepime	28.0	65.1	2.0	4.70	13	30.0
Imipenem	43.0	100.0	0.0	0.0	0	0.0
Chloramphenicol	0.0	0.0	12.0	27.9	31	72.1
Tetracycline	0.0	0.0	3.0	6.98	40	93.0
Gentamicin	19.0	44.2	7.0	39.5	7	16.3
Ofloxacin	0.0	0.0	22.0	51.2	21	48.8
Perfloxacin	11.0	25.6	10.0	3.3	22	51.2
Ciprofloxacin	17.0	39.5	8.0	18.6	18	41.9

Table 3 and 4 present the result of the minimum inhibitory concentration and the outcome of the addition of 10µg/mL of CCCP to the observed MIC respectively. It was observed that the MICs of most *Pseudomonas aeruginosa* strains tested decreased in the presence of the efflux pump inhibitor (Table 4). All the *P. aeruginosa* isolates cultured in Muller Hinton broth containing the CCCP 10µg/mL without the antibiotic, showed growth of bacteria, indicating that the CCCP did not have an antibacterial effect itself. A lowered MIC in the presence of CCCP was interpreted as efflux system while higher MIC or no effect of the MIC after the incorporation of CCCP was taken to be lack of efflux system. The results therefore emphasize that drug efflux pump systems contributed to resistance of ciprofloxacin, cefepime, and imipenem. However, gentamicin resistance observed in this study was thought not to be due to efflux pump system. This is because the incorporation of the CCCP had no effect on the previous MIC value.

Table 3: MICs of different antibiotics for *Pseudomonas aeruginosa* isolates (N= 43)

Antibiotics	Break point (µ/ml)	Number of isolates that had their MICs in each concentration										
		1	2	4	8	16	32	64	128	256	512	>512
Ampicillin	8	0	0	0	0	0	7	6	8	3	2	17
Cefuroxime	16	0	0	0	0	10	8	16	2	1	5	1
Cefriazone	8	0	0	0	3	6	7	1	3	5	18	0
Cefotaxime	8	0	0	0	0	0	8	10	12	6	7	0
Cefepime	8	0	0	8	8	2	10	3	0	0	0	0
Imipenem	4	12	8	20	3	0	0	0	0	0	0	0
Chloramphenicol	8	0	0	0	0	12	6	2	16	4	3	0
Tetracycline	4	0	0	0	3	18	6	3	10	3	0	0
Gentamicin	4	0	0	19	17	6	1	0	0	0	0	0
Ofloxacin	2	0	0	22	18	1	2	0	0	0	0	0
Perfloxacin	2	7	4	10	18	3	1	0	0	0	0	0
Ciprofloxacin	1	17	18	12	6	0	0	0	0	0	0	0

Table 4: Effect of adding CCCP on antibiotic resistance pattern of *Pseudomonas aeruginosa* isolates

Isolates no	MIC of Ciprofloxacin in the presence of 10 µg/mL CCCP		MIC of Cefepime in the presence of 10 µg/mL CCCP		MIC of Gentamicin in the presence of 10 µg/mL CCCP		MIC of Imipenem in the presence of 10 µg/ML CCCP	
1	16	14	64	64	32	32	8	8
2	16	4	64	32	16	16	4	2
3	16	16	32	32	16	32	4	2
4	8	8	64	64	8	8	4	4
5	8	8	32	16	8	16	4	4
6	4	4	16	8	4	32	4	2
7	2	1	8	8	4	32	4	2
8	2	2	8	8	4	32	4	2
9	1	1	64	32	16	16	4	1
10	1	1	4	4	16	32	4	2
11	4	4	4	2	16	64	2	1
12	4	2	4	2	16	32	2	1
13	8	4	4	2	16	16	1	1
14	8	2	4	2	16	32	1	1
15	8	1	4	1	16	32	1	1

DISCUSSION

The emergence of multiple antibiotic resistance in *Pseudomonas aeruginosa* and the indiscriminate use of antibiotics contribute to the dissemination of resistant bacteria in the environment which brings about problem in therapy thereby generating serious public health issue. The ubiquitous presence of *P. aeruginosa,* its adaptable nature and their multi drug resistance characteristic have been documented [Lambert, 2002; Yetkin *et al.,* 2007].

The relative higher occurrence of *Pseudomonas aeruginosa* in environmental specimens compared to other *Pseudomonas* species could be due to the possession of additional genetic capacity compared with other species [Lambert, 2002; Fair and Tor, 2014].This corroborates with the finding of Jefferies *et al.* [2012] who reported this organism as the most common gram-negative bacteria causing nosocomial infections. Fair and Tor, [2014] who itemized some factors that increase *P. aeruginosa* resistance thereby making its infection complicated and life threatening.

The observation of imipenem as the most efficacious in this study could mean that *P. aeruginosa* from the study area possess the oprD porin as it has been reported that loss of oprD porin is responsible for *P. aeruginosa* resistance to imipenem. Reason is that this bacterium needs the porin to cross the outer membrane Livermore, [2001] and oprD has be identified to be a specialized porin with a specific role in the uptake of positively charged amino acids such as lysine. Also, loss of this porin has been reported by Livermore, [2001] to increase the minimum inhibitory concentration from 1-2 to 8-32mg/L and that 17% rate of resistance has been observed during treat-

ment. This finding agrees with the findings of Kohler *et al.* [1997], who reported the sensitivity of *P. aeruginosa* to imipenem, pefloxcin and ciprofloxacin.

The susceptibility of *P. aeruginosa* to cefepime and gentamicin besides imipenem could be due to the fact that these antibiotics are among the newest set of antibiotics in the market and their high cost may probably limit its use [Walaa *et al.,* 2018]. This also translates that these antibiotics have not been abused in the study area, hence are still very active for the treatment of infection caused by this organism. According to Livermore, [2001 extensive use of antibiotics to treat of *P. aeruginosa* has lead to selective pressure that encourages the development of resistance.

Ampicillin and cefuroxine, followed by tetracycline, ceftriazone, chloramphenicol and cefuroxime however, were the most resisted antibiotics by the isolates of *P. aeruginosa* and over 40% exhibited multi-drug resistance to more three or more antibiotics. This observation is parallel to that of Kohler *et al.,* [1997]; who regarded over use of these antibiotics as a major factor for the emergence and dissemination of multi- antibiotics resistance strains of several microbes. The fact that 41.9% of these isolates were multi-drug resistant showed that these isolates might have harbor resistance R- plasmids [Olayinka *et al.,* 2009].

In this study efflux pump inhibitors reduced the MIC of *Pseudomonas aeruginosa* to some antibiotics. It was reported that addition of CCCP at concentration of 25µg/mL decreased the MIC of different biocides from 2 to 25µg/ in *Acinetobacter baumanni* [Rajamohan *et al.,* 2010]. The lowering of the MIC of the isolates in the presence of car-

bonyl cyanide 3-chlorophenyl hydrazone clearly demonstrates that the resistance observed in this study is intrinsic resistance that resulted from multi-drug efflux pumps within the bacterial chromosome and also as a result of the low permeability of the bacterial cellular envelopes [Olayinka *et al.,* 2009]. The result of this study disagrees with the works of [Ardebili *et al.,* 2014] who observed that 86 % of the isolates became less resistant (2 to 64 folds) to ciprofloxacin in the presence of efflux pump inhibitors. Also, [Lin *et al.,* 2009] reported that ciprofloxacin susceptibility of most isolates increased in the presence of CCCP by 2 to 8 folds. This efflux pump according to [Li *et al.,* 2003] is not supported by the molecular structure of these pumps. The fact that a more active multi-drug efflux system was found against cefepime, gentamicin and imipenem may be due to the presence of multi-drug resistance pump (MDR) substrates and these pump substrates are molecules that are both lipophilic and cationic [Kohler *et al.,* 1999c]. Although, acquired resistance by active efflux generally results from increased expression of MDR pumps, some MDR pumps are expressed at sufficient level in wild type, while no mutants' bacteria are affected by their intrinsic susceptibility [Radostis *et al.,* 2004]. Results of this study have shown that the environmental sources examined habour varied degree of multidrug resistant efflux mediated *Pseudomonas aeruginosa* which is a big threat to the community and its environs.

CONCLUSION

In conclusion, the intrinsic resistance of *Pseudomonas aeruginosa* to multiple antibiotics in this study could be said to be as a result of efflux of these components from the bacterial cell. It can therefore be encouraged and be thought by health care profes-

sionals so as not to enhance the epidemic rate of multi-drug resistance bacteria in order to antagonize the trend of such isolates so as not to be the source of hospitalization in the vicinity.

Conflict of Interests
Authors have declared that no competing interests exist.

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