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PREVALENCE AND ANTIMICROBIAL RESISTANCE OF *PSEUDOMONAS AERUGINOSA* RECOVERED FROM ENVIRONMENTAL AND CLINICAL SOURCES IN BENIN CITY, NIGERIA

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ABSTRACT

The increasing rate of resistance to antimicrobial agents is a public health challenge and *Pseudomonas aeruginosa* is known to have recalcitrant resistance to several antibiotics. In this study, we characterized antimicrobial resistance and multiple antibiotic resistance (MAR) index of *P. aeruginosa* of environmental and clinical origin. A total of 240 samples were examined, of which 120 each were of environmental and clinical settings. Bacteriological analysis, antimicrobial sensitivity and MAR were performed on the isolates. The results revealed that of the 120 clinical matrixes evaluated for the presence of *P. aeruginosa*, 54.16% (65/120) were positive for *P. aeruginosa*. There were significant differences among the clinical samples ($p < 0.05$) in prevalence. The highest isolation rate 27.7% was observed in wound samples and the least 10.8% in urine. For environmental samples, 45.83% (55/120) were positive for *P. aeruginosa*. There was a highly significant difference among the environmental samples ($p < 0.01$) in prevalence. All positive isolates were resistant to cefuroxime (100%) and amoxicillin (100%). Most were also resistant to nalidixic acid (88%), cotrimoxazole (86%) and ciprofloxacin (85%). There was high significant difference in the resistance patterns of the isolates at $p<0.001$. All the isolates were multi-resistant revealed by the high MAR index profile. The multi-resistance exhibited by *P. aeruginosa* further confirms the call for integrated approaches to combat bacterial antibiotic resistance.

Keywords: *Pseudomonas aeruginosa,* Antibiotics resistance, Multidrug resistance

INTRODUCTION

Pseudomonads are non-fermentative, aerobic, Gram-negative bacilli that are ubiquitous in water, soil, and other humid environs. *Pseudomonas aeruginosa* is frequently ascribed with ailments in humans, where it functions as nosocomial microbial pathogen with a functional systematic approach to initiate infections in virtually any tissue or organ, particularly in immuno-compromised patients or in elderly individuals. The ability of *P. aeruginosa* to initiate diseases is increased by both acquired and innate resistance to a significant number of disinfectants and antimicrobials, ability to strive in a broad range of environmental conditions and virulence determinants (Kerr and Snelling, 2009).

Pseudomonas aeruginosa thrives not only in normal atmospheres, but also in low-oxygen atmospheres, thus has colonized many natural and artificial environments. It uses a wide range of organic material for food; in animals, its versatility enables the organism to infect damaged tissues or those with reduced immunity. In healthcare settings, the bacterium is regarded as a potent cause of infection in individuals with diverse vulnerability including those receiving intensive care or with burns or neutropenia, bacteremia, wound infections as well as other superficial and systemic infections (Lodise *et al.,* 2007). Amongst these categories of persons, morbidity and mortality attributable to infection of *P. aeruginosa* origin can be on the rise. Managing these ailments could prove difficult as *P. aeruginosa* is adversely resistant to broad numbers of antimicrobials. In addition, treatment regimen has become difficult due to emergence and spread of resistant strains of the organism (Kollef *et al.,* 2008).

Literature have reported a number of outbreaks of *P. aeruginosa* affecting intensive care units (ICUs) which have established the potential for *Pseudomonas* spp., to institute microbial reservoirs in the healthcare environment (Hota *et al.,* 2009; Breathnach *et al.,* 2012). In contrast with other environmental pathogens of public health importance, *P. aeruginosa* flourishes in the environment over an extensive range of temperatures with an overwhelming capacity at manipulating and flourishing in relatively nutrient-poor surroundings (Igbinosa and Obuekwe, 2014). Its capsular polysaccharide facilitates it to

stick to surfaces. Contrariwise, when good environmental conditions prevail, the cells embark on the exponential phase rapidly on surfaces in connection with water carriage systems to form biofilms (Loveday *et al.,* 2014).

A notable advancement is carbapenemases-producing ability by some strains of *P. aeruginosa* origin. As a consequence of these challenges, it would seem reasonable to identify sources and reservoirs of this bacterium so as to prevent the acquisition of the bacterium by both hospitalized and un-hospitalized persons. Numerous reports of ailments from *P. aeruginosa* outbreaks have been attributed to environmental sources (Jefferies *et al.,* 2012). However, the routes of such sources in sporadic pseudomonad ailments are well less understood. Nonetheless, there is evolving substantiation from potential studies to suggest that environmental sources may have significance in the epidemiology of sporadic *P. aeruginosa* infections in clinical settings. A better understanding of the antimicrobial resistance profiling of clinical and environmental reservoirs in pseudomonads isolates will permit the development of new strategies and refinement of existing approaches to interrupt transmission from these sources. Hence, this study was aimed at determining the antibiogram profiling of *Pseudomonas aeruginosa* from clinical and environmental sources.

MATERIALS AND METHODS

Collection of Samples

A total of 240 specimens comprising 120 each from clinical and environmental sources were examined. The samples were collected from two government-owned hospitals (University of Benin Teaching Hospital and Central Hospital, Benin); and two privately-owned hospitals (St. Philomena Catholic Hospital, Benin and Faith Mediplex, Benin), in Benin City, Edo State, Nigeria.

The clinical samples were made up of 20 urine samples, sputum, 20 wound swab samples, 20 blood samples, 20 infected ear swab samples and 20 swab samples from the eyes. They were collected from hospitalized and non-hospitalized persons attending the clinics. Ethical clearance was obtained for the collection of clinical samples. The environmental samples were from hospital environment (80), abattoirs (20) and dump sites (20) within Benin City. All samples were collected aseptically.

Isolation of *Pseudomonas aeruginosa*

An aliquot of 1.0 ml of the samples was serially diluted using standardized serial dilution procedures to the order of 10^6. Thereafter, 100 µl from respective diluent (10^2-10^6) were inoculated on cetrimide agar and incubated at 37 °C for 18-24 h. Discrete green colonies on the cetrimide agar were sub-cultured and purified on cetrimide agar and incubated at 37 °C for 18-24 h. Thereafter, colonies were purified on nutrient agar and incubated at 37 °C for 24-48 h, and stored using agar slants at 4 °C until ready for use in subsequent analysis.

Phenotypic Identification of *Pseudomonas aeruginosa*

The presumptive identified *Pseudomonas aeruginosa* isolates were further subjected to haemolysis on blood agar, catalase, urease, indole, Gram reaction, citrate, oxidase, oxidation/fermentation reaction, and motility test as described by Cheesbrough (2005).

Antibiotic Susceptibility Testing

Antibiotics susceptibility testing was carried out using the disc diffusion (Kirby-Bauer) technique, as recommended by standard guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2014). Briefly, a single inoculum of each bacterial isolates was emulsified in 5.0 ml sterile normal saline in Bijou bottles to make a lawn of bacteria. Sterile cotton swabs were dipped into the standardized solution of bacterial cultures and used to inoculate Mueller-Hinton agar plates. Thereafter, antibiotic discs (Mast Diagnostics, Merseyside, United Kingdom) containing the antibiotics: amikacin (30 µg), gentamicin (10 µg), streptomycin (10 µg), tobramycin (10 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), amoxicillin (10 µg), imipenem (10 µg), meropenem (10 µg), cotrimoxazole (25 µg), aztreonam (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), nalidixic acid (30 µg) and tetracycline (30 µg), were placed on the plates. Discs were placed at least 15 mm apart and from the edges of the plates to prevent

overlapping of inhibition zones. The plates were incubated at 37 °C for 18-24 h, after which zones of inhibition (millimetres) were measured to determine sensitivity, intermediate or resistance profile. The zones were interpreted according to the standards of Clinical and Laboratory Standards Institute (CLSI, 2014).

Multiple Antibiotic Resistances (MAR) Index

The multiple antibiotic resistance (MAR) index was determined for each isolate by dividing the number of antibiotics to which the isolate is resistant by the total number of antibiotics tested (Krumpernam, 1983).

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

MAR index higher than 0.2 identifies that organism to have originated from high-risk sources of contamination, where antibiotics are often used (Krumpernam, 1983).

Statistical Analysis

All data were tabulated and analyzed using SPSS, version 12.0. Qualitative variables were expressed by percentages and compared using charts and chi-square test. A p-value < 0.05 was considered statistically significant.

RESULTS

A total of 120 clinical matrixes evaluated for the presence of *P. aeruginosa*, 54.16% (65/120) were positive for *P. aeruginosa*. There were significant differences among the clinical samples ($p < 0.05$) in prevalence. The highest isolation rate 27.7% was observed in wound samples and the least 10.8% in urine (Table 1). The 120 environmental samples evaluated revealed that 45.83% (55/120) were positive for *P. aeruginosa*. There was a highly significant difference among the environmental samples ($p < 0.01$) in prevalence (Table 2).

Table 1: Prevalence of *P. aeruginosa* from Clinical Samples

Clinical Specimens	Total no of Samples	Positive Samples for *P. aeruginosa* (%)
Sputum	20	9 (13.8)
Wound	20	18 (27.7)
Urine	20	7 (10.8)
Blood	20	10 (15.4)
Ear	20	13 (20.0)
Eyes	20	8 (12.3)
Total	120	65 (100)

$p < 0.05$

Table 2: Prevalence of *P. aeruginosa* from Environmental Sources

Environmental Sites	Total no of Samples	Positive Samples for *P. aeruginosa* (%)
Abattoir	20	9 (16.4)
Dump sites	20	16 (29.1)
Catheter tips	20	11 (20.0)
Hospital walls	20	6 (10.9)
Hospital beds	20	5 (9.1)
Hospital sinks	20	8 (14.5)
Total	120	55 (100)

$p < 0.01$

The antibiotic susceptibility patterns of clinical and environmental isolates are shown in table 3. All positive clinical isolates were resistant to cefuroxime (100%) and amoxicillin (100%). Most of the clinical isolates were also resistant to nalidixic acid (88%), cotrimoxazole (86%) and ciprofloxacin (85%). There was a very high significant difference in the resistance patterns amongst the clinical isolates at $p<0.001$. The clinical isolates were most sensitive to imipenem (91%), amikacin (78%), ceftazidime (75%) and meropenem (72%) (Table 3).

All environmental isolates were resistant to cefuroxime (100%), amoxicillin (100%), tetracycline (100%) and chloramphenicol (95%). There was a very high significant difference in the resistance patterns of the environmental isolates at $p<0.001$. Some of the environmental isolates were however sensitive to ceftazidime (78%), meropenem (77%), amikacin (73%) and imipenem (71%) (Table 3).

Table 3: Antibiotic Susceptibility of *P. aeruginosa* Isolates from Clinical and Environmental Sources

Antibiotic Class	Antibiotics	Clinical (n=65)			Environmental (n=55)		
		R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Aminoglycosides	Amikacin (AK)	13(20)	1(2)	51(78)	14(25)	1(2)	40(73)
	Gentamicin (GM)	46(71)	8(12)	11(17)	24(44)	11(20)	20(36)
	Streptomycin (S)	40(62)	10(15)	15(23)	44(80)	11(20)	0(0)
	Tobramycin (TN)	51(78)	8(12)	6(15)	35(64)	13(23)	7(13)
Cephalosporines	Ceftazidime (CAZ)	13(20)	3(5)	49(75)	10(18)	2(4)	43(78)
	Ceftriaxone (CRO)	31(48)	25(38)	9(14)	31(56)	16(29)	8(15)
	Cefuroxime (CXM)	65(100)	0(0)	0(0)	55(100)	0(0)	0(0)
Penicillins	Amoxicillin (A)	65(100)	0(0)	0(0)	55(100)	0(0)	0(0)
Carbapenems	Imipenem (IMI)	4(6)	2(3)	59(91)	10(18)	6(11)	38(71)
	Meropenem (MEM)	15(23)	3(5)	47(72)	10(18)	3(5)	42(77)
Follate Inhibitors	Cotrimoxazole (TS)	56(86)	3(5)	6(9)	48(87)	2(4)	5(9)
Monobactams	Aztreonam (ATM)	44(68)	9(14)	12(18)	46(84)	4(7)	5(9)
Phenicols	Chloramphenicol (C)	34(52)	15(23)	16(25)	52(95)	3(5)	0(0)
Quinolones	Ciprofloxacin (CIP)	55(85)	4(6)	6(9)	35(64)	7(13)	13(23)
	Ofloxacin (OFX)	44(68)	11(17)	10(15)	34(62)	5(9)	16(29)
	Nalidixic acid (NA)	57(88)	4(6)	4(6)	49(89)	4(7)	2(4)
Tetracyclines	Tetracycline (T)	26(40)	23(35)	16(25)	55(100)	0(0)	0(0)

Legend: % S- Sensitivity rate; % I- Intermediate rate; % R- Resistance rate

The resistant percentage profile of *P. aeruginosa* of the clinical and environmental isolates to each of the antibiotics tested is shown in figure 1. There was no significant difference in resistance pattern between the clinical and environmental isolates to the following antibiotics: amikacin, streptomycin, tobramycin, ceftazidime, ceftriaxone, cefuroxime, amoxicillin, meropenem, cotrimoxazole, aztreonam, ciprofloxacin, ofloxacin and nalidixic acid, ($p>0.05$). There was high significant difference in resistance pattern to gentamicin, imipenem, chloramphenicol and tetracycline at $p<0.01$ significance level.

The multiple antibiotic resistance (MAR) indices of the evaluated clinical and environmental isolates are shown in figure 2. Isolates from blood, wound, catheter and dump sites revealed very high MAR index values (>0.8), indicating high-risk sources of contamination and possible transmission of infection.

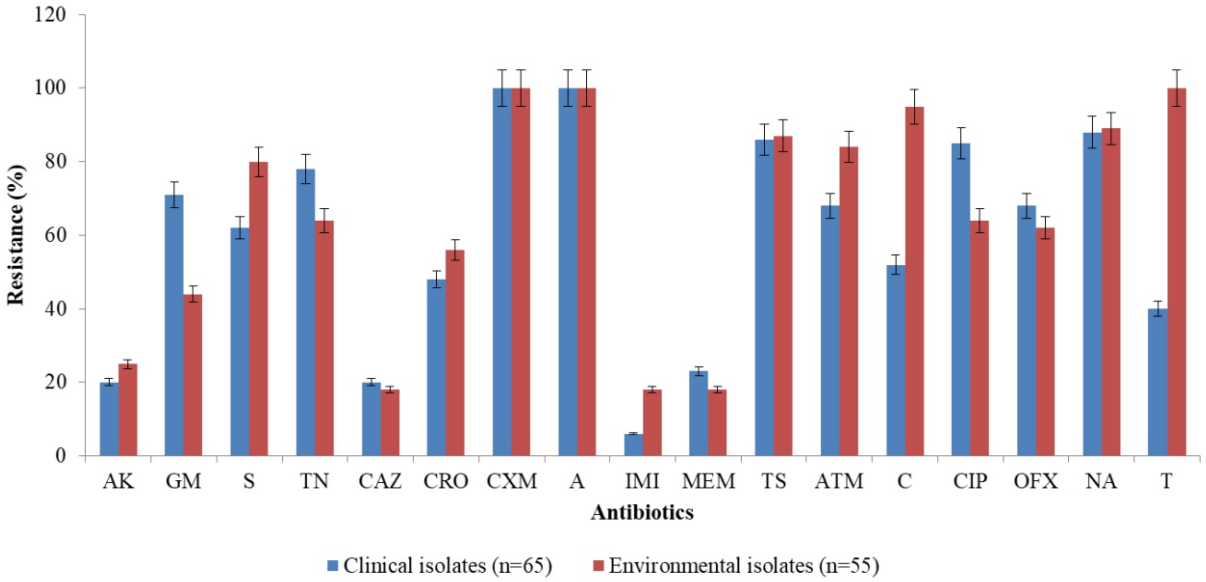

Figure 1: Percentage Profile of *P. aeruginosa* Resistant from Clinical and Environmental Isolates
Legend: AK=Amikacin, GM=Gentamicin, S=Streptomycin, TN=Tobramycin, CAZ=Ceftazidime, CRO=Ceftriaxone, CXM=Cefuroxime, A=Amoxicillin, IMI=Imipenem, MEM=Meropenem, TS=Cotrimoxazole, ATM=Aztreonam, C=Chloramphenicol, CIP=Ciprofloxacin, OFX=Ofloxacin, NA=Nalidixic acid, T=Tetracycline.

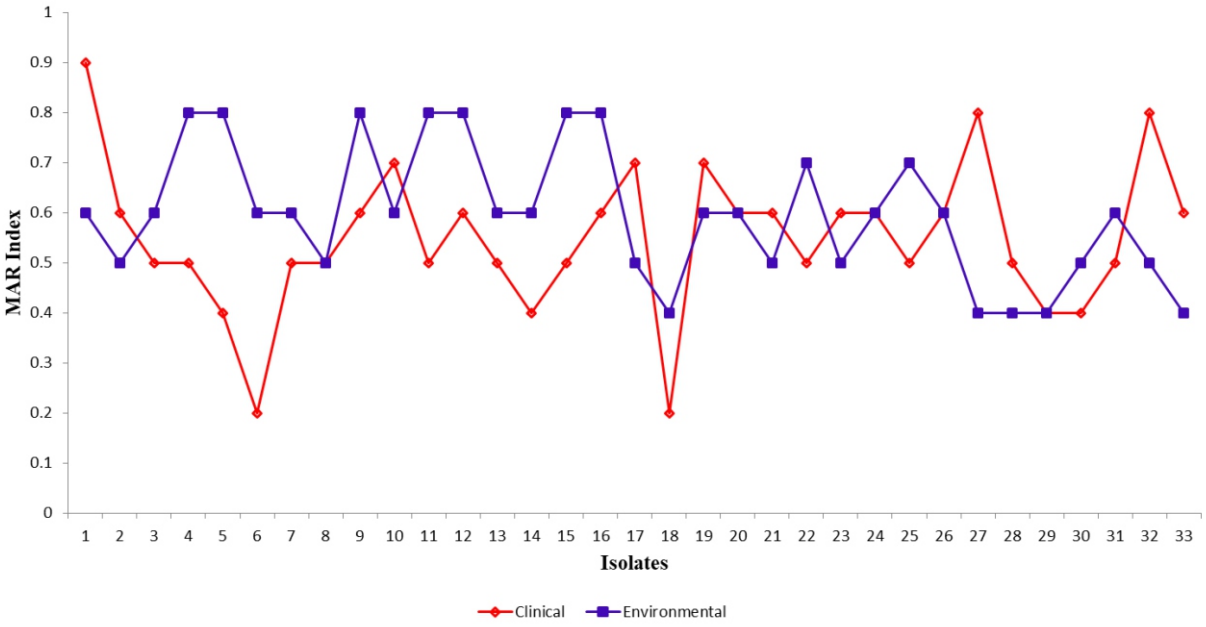

Figure 2: Multiple Antibiotic Resistance (MAR) Index Profile from Clinical and Environmental Isolates of *Pseudomonas aeruginosa*

DISCUSSION

Pseudomonas aeruginosa remains a significant nosocomial pathogen associated with morbidity and mortality, particularly for immuno-compromised individuals and vulnerable patients on intensive care units (Kerr and Snelling, 2009). Findings from this study have stressed the important potential clinical (sputum, wound/burns, urine, blood, ear and eyes infection) and environmental sources (abattoir, dumpsites, catheter tips, hospital walls, hospital beds and hospital sinks) as a potential reservoir of antibiotic-resistant *P. aeruginosa*.

Earlier reports in Nigeria showed varying isolation rates of this organism from clinical samples. These include those of Odusanya (2002) who reported 4.6% for urine and 16.3% from wound infections, prevalence of 11.1% in open musculoskeletal injuries (Akinyola and Ako-Nai, 2005); 41.9% and 39.35% from ear and wound swab respectively (Ogbolu *et al.*, 2008). From this study also, the highest isolation rate of 90% was observed in wound samples and the least 35% in urine ($P > 0.05$). This represents a major public health hazard for both hospital and community acquired infection especially for surgical wound contamination.

From the environmental samples, the results indicated that 45.80% were positive for *P. aeruginosa*. The prevalence of the organism in clinical specimens (54.2%) was not significantly different ($P > 0.05$) from that of environmental specimens (45.80%). High prevalence was observed in dumpsites (80%) and catheter tips (55%). The organism had been isolated from catheter tip (Aibinu *et al.*, 2007; and Mansour *et al.*, 2013). Previous reports of isolation from dump sites include the works of Achudume and Olawale (2007), and Oviasogie *et al.*, (2010). The high prevalence of the organism in dumpsites in this study is a potential threat to health in the case of indiscriminate dumping of wastes. The organism was also isolated from abattoirs, which is in agreement with a study conducted by Igbinosa and Obuekwe (2014) on abattoir environment around Benin City.

As regards hospital environment, the range of reservoirs in healthcare environments from which *P. aeruginosa* has been isolated is wide ranging from potable water, tap traps, showers, disinfectants, respiratory therapy equipment, mop heads, endoscopes, urometers, water baths, hydrotherapy pools, infant feeding basins, bathing basins, bath toys to cleaning equipment (Rogues *et al.*, 2007). Several properties of *P. aeruginosa* contribute to persistence in the hospital environment. The organism is inherently resistant to several disinfectants such as biguanides and quaternary ammonium compounds through the mechanism of multidrug efflux pumps. Also, the ability of the organism to form biofilm on inanimate surfaces also favours disinfectant resistance as well as impeding physical removal. The type III secretion system to kill free-living amoeba which graze on environmental bacteria also contributes to its persistence in moist environments (Matz *et al.*, 2008).

In this study, a good percentage of the clinical and environmental isolates were resistant to majority of the antibiotics: penicillins (100%), the cephalosporins: ceftriaxone and cefuroxime (48% - 100%) and sulphamethoxazole (86% - 87%) in agreement with reports of other authors (Li *et al.*, 2010; and Ashish *et al.*, 2011). The data showed the highest antibiotic resistance to amoxycilin and cefuroxime, and the lowest resistance to imipenem, meropenem, amikacin and ceftazidime, in both clinical and environmental isolates. This correlated with the works of Mahmoud *et al.* (2013) who reported from their research that amikacin and imipenem were the most effective drugs against *P. aeruginosa*. Haleem *et al.* (2011) also reported that clinical and environmental isolates of *P. aeruginosa* was 100% resistance to cefotaxime, chloramphenicol, penicillin, ampicillin, doxycycline, erythromycin, tetracycline and cloxacillin. High resistances of these isolates to the β- lactam antibiotics may be due to production of beta-lactamase enzymes that breakdown the beta-lactam ring. Pirnay *et al.* (2005) reported that *Pseudomonas* species were naturally resistant to the penicillins, cephems and rifampin because they have relatively impermeable membrane, inducible efflux systems and a chromosomally encoded inducible β-lactamase.

The resistance rate to imipenem was 6% and 18%, clinical and environmental isolates respectively. A decline in the resistance to imipenem could be attributed to the restricted use of this antibiotic. The result of this study showed that there is marked increase in resistance to the quinolones (ciprofloxacin, ofloxacin and nalidixic acid). Nalidixic acid had the highest resistance (88% and 89%) and was closely followed by ciprofloxacin (85% and 64%) and ofloxacin (68% and 62%) for clinical and environmental isolates respectively. This result implies that quinolones alone cannot be depended upon as an antipseudomonal antimicrobial in this environment. In previous studies by Aibinu *et al.* (2007), *Pseudomonas aeruginosa* strains were found to be highly

susceptible to the quinolones (96%). Due to the increasing resistance to nalidixic acid in many hospitals, its empirical usage is either banned or restricted, to bring the developing resistance rates under control (Algun *et al*., 2004).

It was interesting to note that all the environmental isolates were resistant to tetracycline. The remarkable multi-resistances to tetracycline could be that this antibiotic is highly misused because of constant and indiscriminate usage in our environment ( ), and an intrinsic and acquired resistance mechanism caused mainly by an active efflux system, which efficiently expels the compound from the cell ( ). The resistance observed in *P. aeruginosa* to a number of antibiotics could be as a result of gene transfer into the hospital environment, which is a common nosocomial occurrence and incessant use of antibiotics. From this study, there was no significant difference between resistance patterns of clinical and environmental isolates (P>0.05) to majority of the antibiotics.

All the tested isolates in this study showed multiple antibiotic resistances (MARs) ranging from four to 16 antibiotics distributed among three to seven classes. The MAR indices were higher than the 0.2 limit in almost all tested isolates. This correlates with studies of Odjadjare *et al*. (2012) who reported MAR of five to 11 antibiotics. Navon-Venezia *et al*. (2005) observed that MAR bacterial strains may also arise as a result of unrelated mechanisms accumulating sequentially in an organism. The observation indicates that isolates in this study originated from high risks source(s) of contamination. Multi-drug resistance in environmental isolates might be linked to the uncontrolled disposal of antibiotics and chemicals into the environment creating a selective pressure on these drugs. The use of antibiotics in hospital and the community at large serves as a major selective pressure for antibiotic resistant bacteria ( ).

CONCLUSION

This study has established that *P. aeruginosa* is highly ubiquitous and can be isolated from diverse clinical and environmental sources at different ecological habitat. The isolates were resistant to majority of the anti-pseudomonad drugs tested, and sources originated from high risk sources of contamination. The diversity and prevalence of resistance phenotypes in clinical and environmental sources inspire hypotheses about the native roles of resistance gene element in natural microbial communities.

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