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RISK SURVEILLANCE OF MULTIDRUG RESISTANT *PSEUDOMONAS AERUGINOSA* IN WATER AND PLASMID RELATEDNESS WITH CLINICAL STRAINS IN ABEOKUTA, SOUTHWEST NIGERIA

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ABSTRACT

Pseudomonas aeruginosa as an opportunistic pathogen has been a subject of investigation due its intrinsic drug resistance. Its frequent presence in drinking, domestic and recreational water highlights its significance to public health. This study was aimed at risk surveillance of multidrug resistant environmental *P. aeruginosa* in water and their plasmid relatedness with clinical strains in Abeokuta, southwestern Nigeria. A total of forty-one (41) strains with prevalence: well water (29.3%); swimming pool (22.0%) hospital storage tank (19.5%); tap water (14.6%); sachet water (12.2%); and bottled water (2.4%) respectively were isolated from two hundred and eighty eight (288) water samples and were compared with 43 clinical strains from wound (37.3%), blood (11.6%), ear swab (20.9%) and urine (20.9%) and eye swab (9.3%). Both environmental and clinical strains were all multidrug resistant, though with different plasmid profile. Plasmid with molecular weight size of 2010bp was detected in only 1 (2.5%) out of the 41 environmental strains as against 9 (20.93%) of the 43 clinical strains having between 22520-23130bp molecular weight. All strains harboring plasmid were resistant to varied types of more than seven drugs out of the eleven tested (gentamycin 10µg, erythromycin 15µg, ampicillin 10µg, augmentin 10µg, cotrimoxazole 25µg, tetracycline 30µg, streptomycin 10µg, ciprofloxacin 5µg, cloxacillin 5µg, amoxicillin 25µg, and cefuroxime 30µg). Strains without plasmid were also multidrug resistant. This finding would be important in the control of multidrug resistant *Pseudomonas aeruginosa* infection in Nigeria.

KEY WORDS: *Pseudomonas aeruginosa*; multidrug resistance; drinking water, recreational water; Plasmid profile

INTRODUCTION

Pseudomonas aeruginosa is capable of causing a variety of life threatening human infections (Aliaga *et al*., 2002). Infections caused by *Pseudomonas aeruginosa* are particularly problematic because the organism is inherently resistant to many drug classes and is able to acquire resistance to all effective antimicrobial drugs (Goldberg, 2000).

Pseudomonas aeruginosa has been an important pathogen in nosocomial infections (Rutala and Weber, 1997; Blondel-Hill *et al*., 2009). Water is one of the principal agents of cross contamination in the clinical environment. It is associated with water taps, sinks, traps, and bath toys (Buttery *et al.,* 1998; Reuter *et al*., 2002). *Pseudomonas aeruginosa* can also contaminate catheters and intravenous lines

(IVs) (Edgeworth *et al*., 1999). Ventilator-associated pneumonia (VAP) is frequently caused by *P. aeruginosa (*Bergmanns *et al.,* 1998).

Frequent presence of *P. aeruginosa* in recreational and drinking water also highlights its significance to public health. Zichichi *et al*. (2000) reported *P. aeruginosa* in folliculitis after shower/bath exposure and Barben *et. al*. (2005) reported its occurrence in bathroom of patients with cystic fibrosis. *Pseudomonas aeruginosa* is also naturally found in many types of drinking water (Hardalo and Edberg, 1997; Hirulkar and Soni, 2011), drinking water biofilms (Moritz *et al.,* 2010; Waines *et al*., 2011), hot tubs (Dulabon *et al.,* 2009), mobile bathing service (Sakurai-Komada *et al*., 2006) and swimming pools (Mena and Gerba, 2009; Tirodimos *et al*., 2010). Outbreaks associated with contaminated water outlets in hospitals are well documented (Bert *et al*., 1998; Pitten *et al*., 2001; Aumeran *et al*., 2007; Cervia *et al*., 2008; Kohlenberg *et al*., 2010; Nagao *et al*., 2011).

Studies on *Pseudomonas aeruginosa* are very important because of the organism's ever growing multidrug resistance (Micek *et al*., 2005; Lutz and Lee, 2011). This type of study particularly, surveillance in drinking, domestic, recreational and hospital storage water, and relationship with clinical infections is rare in Nigeria. This study was designed to survey different water sources for risk of multi-drug resistant *P. aeruginosa* in Abeokuta, south western Nigeria and to determine strains relatedness with clinical isolates in terms of plasmid DNA profile.

MATERIALS AND METHODS

Collection of Water Samples

A total of two hundred and eighty eight (288) water samples were collected from 6 different sources ('Sachet water', 'bottled water', swimming pool, hand-dug well, hospital storage tank and community tap water) in Abeokuta, Nigeria at weekly intervals during the period of 6 months (October 2011-March 2012). Samples were collected into 250 ml sterile glass bottles during the day between 9:00 hrs and 13:00 hrs and were transported to the laboratory in ice box; microbiological analysis was performed within 4 h of collection.

Isolation of Pseudomonas aeruginosa from water samples

ASTM International Standard Test Method for Isolation and Enumeration of *Pseudomonas aeruginosa* from Water [D 5246 - 92] (2004) was adopted for this study. One hundred milliliter (100 ml) of each water sample was filtered using membrane filtration technique using a sterile 0.45-μm-pore-size, 47 mm diameter cellulose membrane filter (Millipore, Bedford, MA, USA). The filters were then seeded onto Centrimide agar and incubated at 40 ^{o} C for 24 h.

Pseudomonas aeruginosa from clinical samples

P. aeruginosa clinical strains isolated from urine, eye, wound, blood and ear samples, collected as part of routine diagnostic test at the medical laboratory of two hospitals in Abeokuta, Nigeria were obtained for this study after due ethical approval from the Human Research Ethics Committees.

Confirmation of Pseudomonas aeruginosa isolates

Isolated pure cultures of bacteria were grown on Nutrient Agar and confirmed as *P. aeruginosa* using Gram staining and biochemical tests. Colonies were identified using the Bergey's Manual of Determinative Bacteriology.

Multi-drug Resistance Testing

Commercially available antimicrobial disc (Abtek Biological Ltd UK) was used to determine the drug sensitivity and resistance pattern of the isolates to the following: Gentamycin (GEN) 10µg, Erythromycin (ERY) 15µg, Ampicillin (AMP) 10µg, Augmentin (AUG) 10µg, Cotrimoxazole (COT) 25µg, Tetracycline (TET) 30µg, Streptomycin (STR) 10µg, Ciprofloxacin (CIP) 5µg, Cloxacillin (CXC) 5µg, Amoxicillin (AMX) 25µg, and Cefuroxime (CXM) 30µg using the Kirby-Bauer disc diffusion method (Bauer *et al*., 1966). The turbidity of the bacterial suspensions was compared with a Macfarland's barium sulfate standard solution corresponding to 1.5 = 10 cfu/ml. The standardized bacterial suspension was inoculated on to Muller Hinton Agar (Lab M Laboratories, Mumbai, India) and left to dry for 10 m, before placing the antibiotic impregnated discs.

After incubation, the diameter of the zone of inhibition were measured and compared with zone diameter interpretative chart (CLSI, 2008) to determine the sensitivity and resistance of the isolates to antibiotics. Isolates resistance to multiple antibiotics were considered for further study.

Plasmid Isolation and Profiling

Pure isolates were inoculated on Mueller Hinton agar and incubated overnight. Plasmid isolation was conducted using a highly pure isolation kit (Zyppy Plasmid Miniprep Kit). Electrophoresis of the DNA was carried out on a 0.8% agarose gel in a 1X concentration of Tris-Borate-EDTA (TBE) buffer at a constant voltage of 100V, 60°C for about 1 h 30 m. After electrophoresis, the gel was photographed under UV illumination. The approximate molecular weights of the plasmid were determined using marker with known molecular weight as standards.

RESULTS

Prevalence of Pseudomonas aeruginosa in water samples

Figure 1 shows the prevalence and percentage distribution of *Pseudomonas aeruginosa* in different water samples. Out of two hundred and eighty eight (288) water sampled for *P. aeruginosa*; positive results were recorded in: 1 (2.4%) bottled water; 5(12.2%) sachet water; 6(14.6%) tap water; 12(29.3%) well water; 9(22.0%) swimming pool and 8 (19.5%) hospital storage tank.

Pseudomonas aeruginosa load in water samples

Pseudomonas aeruginosa load in the different water samples is shown in Table 1. The highest (18.25×10^3 cfu/ml) mean load of *P. aeruginosa* was observed in well water with values range of 16.0 -20.5× 10^3 cfu/ml while the least (3.5×10^3 cfu/ml) with values range of 2.5- 4.5 × 10^3 cfu/ml was observed in bottled water.

Antibiogram of Pseudomonas aeruginosa strains from water samples

Table 2 shows the antibiogram of *Pseudomonas aeruginosa* strains from water samples. Overall, strains were resistant to Ampicillin (70.3%), Amoxicillin (58.7%), Augmentin (36.6%), Cefuroxime (68.3%), Ciprofloxacin (68.3%), Cloxacillin (90.2), Cotrimoxazole (70.7), Erythromycin (58.5%), Gentamycin (22.0%), Streptomycin (48.8%) and Tetracycline (53.7%).

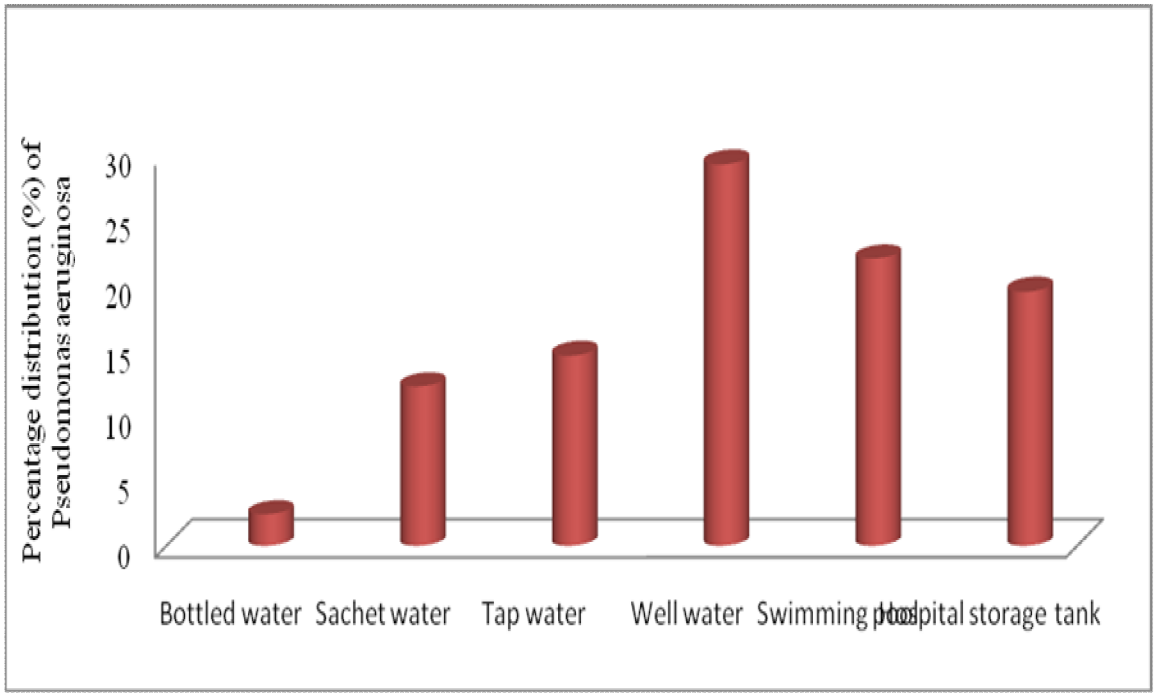

Fig. 1. Prevalence of Pseudomonas aeruginosa in water

Table 1. Mean load of *Pseudomonas aeruginosa* in water samples

Water Sources	Sample size	Mean (x103cfu/ml)	Range (x103cfu/ml)	Standard Deviation (S.D)
Sachet water	32	9.7	8.9-10.5	1.131
Bottled water	32	3.5	2.5-4.5	1.414
Tap water	32	14.5	10.5-18.4	5.587
Well water	32	18.25	16.0-20.5	3.182
Swimming pool	32	17.5	16.5-18.5	1.414
Hospital water	128	11.7	11.0-12.4	0.909

Table 2: Antibiogram of *Pseudomonas aeruginosa* strains from water samples

Antibiotic	AMP (10µg)	AMX (25µg)	AUG (10µg)	CXM (30µg)	CIP (5µg)	CXC (5µg)	COT 25 (µg)	ERY (15 µg)	GEN (10µg)	STR (10µg)	TET (30µg)
Tap water (n=6)	3(50)	6(100)	2(33.3)	3(50)	5(83.3)	6(100)	5(83.3)	4(66.7)	1(16.7)	4(66.7)	4(66.7)
Bottle water (n=1)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)	1(100)
swimming (n=9)	9(100)	0(0)	2(22.2)	5(55.6)	9(100)	9(100)	4(44.4)	5(55.6)	2(22.2)	2(22.2)	3(33.3)
Sachet water (n=5)	4(80)	3(60)	3(60)	4(80)	3(60)	5(100)	5(100)	4(80)	1(20)	3(60)	4(80)
well water (n=12	6(50)	7(58.4)	4(33.3)	11(80)	5(41.6)	9(75)	8(66.7)	6(50)	3(25)	5(41.6)	6(50)
Storage tank (n=8)	6 (75)	5(62.5)	3(37.5)	4(50)	5(62.5)	7(88.5	6(75)	4(50)	2(25)	5(62.5)	4(50)
Overall R (n=41)	29 (70.73)	22 (53.7)	15(36.6)	28 (68.3)	28 (68.3)	37(90.2)	29 (70.7)	24 (58.5)	9 (22)	20 (48.8)	22 (53.7)

Value is showing the number resistant isolates (percentage in parenthesis)

Key: Ampicillin (AMP), Amoxicillin (AMX), Augmentin (AUG), Cefuroxime (CXM), Ciprofloxacin (CIP), Cloxacillin (CXC), Cotri-moxazole (COT), Erythromycin (ERY), Gentamycin (GEN), Streptomycin (STR), Tetracycline (Tet)

Antibiogram of clinical Pseudomonas aeruginosa strains

Out of 43 clinical *P. aeruginosa* isolates, 16 (37.3%) was from wound, 5(11.6%) from blood; 9 (20.9%) each from ear swab and urine and 4 (9.3%) from eye swab. Table 3 shows the antibiogram of clinical *Pseudomonas aeruginosa* strains. *Pseudomonas aeruginosa* strains displayed resistance to: Ampicillin (100%), Amoxicillin (100%), Augmentin (74.4%), Cefuroxime (83.7%), Ciprofloxacin (67.4%), Ofloxacilin (90.7%), Chloramphenicol (81.4%), Cloxacillin (76.7%), Gentamycin (74.4%), Streptomycin (76.7%), and Tetracycline (90%).

Table 3: Antibiogram of clinical *Pseudomonas aeruginosa* strains

SOURCE OF ISOLATE	AMP 10 µg	AMX 25 µg	AUG 10 µg	CXM 30 µg	CIP 5 µg	OFL 10 µg	CHL 30 µg	CAZ 30µg	GEN 10 µg	STR 10 µg	TET 30 µg
Wound (16)	16(100)	16(100)	12(75)	14(87.5)	11(68.75)	14(87.5)	14(87.5)	10(62.5)	13(81.3)	12(75)	15(93.8)
Blood (5)	5(100)	5(100)	5(100)	5(100)	4(80)	5(100)	5(100)	4(80)	2(40)	5(100)	5(100)
Ear swab (9)	9(100)	9(100)	8(88.9)	6(66.7)	6(66.7)	8(88.9)	9(100)	8(88.9)	5(55.6)	6(66.7)	8(88.9)
Urine (9)	9(100)	9(100)	4(44.4)	8(88.9)	5(55.6)	8(88.9)	7(77.8)	7(77.8)	8(88.9)	8(88.9)	8(88.9)
Eye swab (4)	4(100)	4(100)	3(100)	3(100)	3(75)	4(100)	4(100)	4(100)	4(100)	2(50)	4(100)
Total Overall resistant (n=43)	43(100)	43(100)	32(74.4)	36(83.7)	29(67.4)	39(90.7)	35(81.4)	33(76.7)	32(74.4)	33(76.7)	40(90)

Value is showing the number of resistant isolates (percentage in parenthesis)

Key: Ampicillin (AMP), Amoxicillin (AMX), Augmentin (AUG), Cefuroxime (CXM), Ciprofloxacin (CIP), Ofloxacilin (OFL), Chloramphenicol (CHL), ceftazidime(CAZ), Gentamycin (GEN), Streptomycin (STR), Tetracycline (TET)

5. Plasmid profile of environmental and clinical *Pseudomonas aeruginosa*

Plasmid analysis was carried out on the 84 isolates of *P. aeruginosa* from different water sources and clinical samples in Abeokuta. One (2.5%) out the 41 *P. aeruginosa* strains from water, isolated from hospital storage tank had a plasmid with 2010bp weight size, while the remaining 40 strains (97.5%) had no plasmid band. On the other hand, the clinical strains examined showed 20.93% (9 out the 43 strains) having plasmid bands with molecular weight range of 2253-23130bp while the remaining 34(79.07%) had no plasmid bands. All strains with plasmid were resistant to between eight and eleven drugs (Table 4).

Table 4: Multidrug resistance and molecular weight of plasmid bearing strains

Source of isolates	Number of antibiotics resistance	Antibiotic resistance	Plasmid weight
Hospital tank	8	AMX, CXM,CIP, COT, ERY, STR, TET	22910bp
Wound swab	8	AMX, AMP,CHL,STR, GEN, OFL, CAZ, TET	23000bp
Wound swab	11	AMP, AMX, TET, CHL, STR, CXM, GEN, CAZ, OFL, CIP, AUG	22520bp
Wound swab	9	AMP, AMX, TET, CHL, CXM, GEN, CAZ, OFL, CIP	22910bp
Wound swab	10	AMP, AMX, TET, CHL, STR, CXM, GEN, CAZ, OFL, CIP	22730bp
Wound swab	10	AMP, AMX, TET, CHL, STR, CXM, GEN, CAZ, OFL, CIP	22639bp
Wound swab	9	AMX, AMP, AUG, TET, CHL, STR, CXM, CIP, OFL	22798bp
Wound swab	8	AMX, AMP, AUG, STR, CXM, CAZ, CIP, GEN	23000bp
Blood	10	AMP, AMX, AUG, TET, CHL, STR, CXM, GEN, CIP, CAZ	23000bp
Ear swab	8	AMP, AMX, AUG, TET, CHL, GEN, OFL, CIP	23130bp

Key: Ampicillin (AMP), Amoxicillin (AMX), Augmentin (AUG), Cefuroxime (CXM), Ciprofloxacin (CIP), Ofloxacilin (OFL), Chloramphenicol (CHL), Cloxacillin (CXC), Gentamycin (GEN), Streptomycin (STR), Tetracycline (Tet).

DISCUSSION

The prevalence of multi-drug resistant *P. aeruginosa* in drinking, domestic, recreational and hospital water was evaluated in this study. The highest prevalence of 29.3% was observed in well water, a source of drinking water in some households in Nigeria. Prevalence of 12.2%, 14.6%, and 2.4% also occurred in sachet water, tap water and bottled water respectively and these are sources of drinking water that are considered safe for consumption in Nigeria. The isolation of *P. aeruginosa* in drinking water sources is an indication of water quality impairment which has been reported previously in public water supply from different parts of Nigeria (Itah and Akpan, 2005; Popoola *et al*., 2007; Okonko *et al*., 2008; Ajayi *et al*., 2008; Shittu, 2008; Odeyemi *et al*., 2011; Sule *et al*., 2011; Muazu et al., 2012; Ukpong and Peter, 2012; Lateef *et al*., 2012; Adewoye and Adewoye, 2013) and from Neemuch city (Hirulka and Soni, 2011).

Human health risks associated with exposure to *P. aeruginosa* via drinking water ingestion, estimated by Mena and Gerba (2009) showed that the risk of colonization from ingesting *P. aeruginosa* in drinking water is low. The risk is slightly higher if the subject is taking an antibiotic resistant *P. aeruginosa* as observed in this study. The fact that individuals on ampicillin are more susceptible to Pseudomonas gastrointestinal infection probably results from suppression of normal intestinal flora, which would allow Pseudomonas to colonize. The effect of drinking contaminated water results in thousands of deaths every day, mostly in children under five years in developing countries (WHO, 2004). Diseases caused through consumption of contaminated water and poor hygiene

practices are the leading cause of death among children worldwide, after respiratory diseases (WHO, 2003).

In this study, the prevalence of *P. aeruginosa* in swimming pools in Abeokuta was 21.9%. *Pseudomonas aeruginosa* have also been reported from swimming pools in other parts of Nigeria: Portharcourt (Agbagwa and Young-Harry, 2012); Lagos (Bello *et al*., 2012). Outside Nigeria, similar result was obtained by Lutz and Lee (2011) from swimming pools and hot tubs showing 21% for *P. aeruginosa*. A lower prevalence of 16.6% and 17% in swimming pools and recreational waters was observed in the Athens area (Rigas *et al.,* 1998); and 4% in Switzerland (Moore *et al,* 2002). A study from Ireland reported a very high prevalence of *P. aeruginosa* of 38% in swimming pools and 73% of Jacuzzis and spas. Varying rates of *P. aeruginosa* reflects different approaches to the maintenance of swimming pool waters (Barben, 2005). The prevalence of *Pseudomonas* sp. in the pool may be affected by several factors such as the level of free chlorine, the density of use, poor operation, construction and maintenance as well as the presence of large plastic inflatables (Tate *et al*., 2003). Mena and Gerba (2009) also summarized that two routes appear to carry the greatest health risks from contacting water contaminated with *P. aeruginosa* which are skin exposure in hot tubs and lung exposure from inhaling aerosols.

The overall prevalence of drug resistance of *Pseudomonas aeruginosa* isolates was very high in this study. *Pseudomonas aeruginosa* strains from water samples were all multidrug resistance, showing highest resistance to cloxacillin (90.2%) and least to amoxicillin (0.0%). Swimming pool isolates had high resistance (100%) to ciprofloxacin and cloxacillin. A similar result was observed by Lutz and Lee (2011) where 96% of isolates from swimming pool tested were multidrug resistant. Clinical isolate on the other hand, altogether showed greater than 65% resistance to all the antibiotics tested with all isolates showing 100% resistance to ampicillin and amoxicillin.

The occurrence of multidrug resistance *P. aeruginosa* strains observed in this study is similar to other reports across Nigeria (Daini *et al*., 2008; Ogbolu *et al*., 2008; Olayinka *et al*., 2009; Akanji *et al.* (2011), Okesola and Oni (2012), Akingbade *et al*. (2012), Daini and Charles-Onyeaghala (2012), Ehiaghe *et al*., 2013 and other reported hospital outbreaks (Cervia *et al*., 2008; Kohlenberg *et al*., 2010; Nagao *et al*., 2011). In contrary to Gad *et al*. (2007), the clinical isolates of *P. aeruginosa* exhibited higher antibiotic resistance than environmental isolates. Out of all the 84 isolates of *P. aeruginosa* strains, 48.8% were resistant to gentamicin which used to be traditionally considered as a first-line drug against Gram negative bacterial infections in the hospital setting (Oduyebo *et al*., 1997). The use of broad spectrum antibiotics in hospitals exerts selective pressure on bacteria, thereby promoting infections by multi-drug resistant strains (Richard *et al.,* 1994). Resistance to some antibiotics such as ampillicin, amoxillin, streptomycin, tetracycline and cefuroxime showed an increase in comparison with previous studies in different countries (Friedland *et al.,* 2005).

In this study, all isolates with plasmid were resistant to more than seven antibiotics. There is no plasmid relationship between environmental and clinical isolates as only 1 (2.5%) with molecular weight of 22910bp was observed in environmental isolates,

while 9 (20.9%) was observed in clinical isolates (molecular weight ranging from 22010 to 23130bp). This result is in agreement with Salama *et al.* (2012) in a similar study who concluded that the simple use of bacterial protein electrophoresis and plasmid profiling ruled out the relatedness between the clinical and the contaminated water samples. From this study, there is also no relationship between plasmid occurrence and multidrug resistance, for all environmental and clinical isolates without plasmid were also multidrug resistant. This showed that strains inability to possess plasmid does not affect its ability to confer resistances to various antibiotics.

Intrinsic antimicrobial resistance in *P. aeruginosa* is due to low outer membrane permeability, as well as an extensive efflux pump system. Also, some *P. aeruginosa* strains exhibit mutations in fluoroquinolone binding sites, the loss of porin channels, and increased beta-lactamase or cephalosporinase production. Additional resistance mechanisms are acquired from plasmids and multidrug resistance that routinely develops through the course of a treatment regimen (Kato *et al*., 2001; Aeschlimann *et al*., 2003; Lister *et al*., 2009; Strateva *et al.,* 2009). The acquisition of Multidrug resistant *P. aeruginosa* is costly, both in terms of money and human suffering (Carmeli *et al.,* 1999), and results also in higher mortality rate (Harris *et al.,* 1999; Grisaru *et al,* 2000).

CONCLUSION

The prevalence of Multidrug resistant *P. aeruginosa* strain in water is of public health significance, as the source of infection is very important in the curtailment and control of spread of infection. Even though, *P. aeruginosa* has been reported from different clinical and environmental sources, this is the first report on the occurrence of multidrug resistant strains of *P. aeruginosa* from diverse environmental sources in Nigeria. This calls for a serious public health concern, particularly for immune-compromised individuals, such as Human Immunodeficiency Virus (HIV) patients in the current epidemic.

RECOMMENDATIONS

The following recommendations are made from the findings of this study:

Control guidelines on *Pseudomonas aeruginosa* as a potential pathogen in water sources, should be formulated for Nigeria similar to those in developed country for health care providers and for drinking and recreational water.

Other genetic markers apart from plasmid profile should be used to investigate the genetic relatedness of clinical and environmental multidrug resistant *P. aeruginosa*.

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