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Research Article

Antimicrobial Resistance Studies of Plasmid-Borne and Biofilm Forming Pseudomonas species harbouring pelA, pslA and algD Surface Attachment Exopolysaccharide Genes from Water Treatment Plant.

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Abstract
This study was carried out to evaluate antibiotic resistance and surface attachment exopolysaccharide genes in biofilm producing *Pseudomonas* species isolated from aquatic environments. 70 water samples were collected from Ezillo water treatment plant, Ebonyi State, South Eastern Nigeria. Standard techniques were used to characterize the isolates from the water samples while the antimicrobial studies were carried out in accordance to CLSI guidelines via Kirby Bauer disk diffusion method. Biofilm formation was determined by tube binding assay using crystal violet. The presence of surface attachment genes was characterized using 16S rRNA polymerase chain reaction (PCR) analysis. Results revealed high frequency of occurrence of the isolates (81.43%) and biofilm formation (78.95%) from the water treatment plant with a statistical significant difference (P = 0.049). The biofilm isolates were completely resistant to Amoxicillin\Clavulanic acid, Tobramycin, Oxacillin and Aztreonam while showing varied resistance to Imipenem (39.46 %), Ceftaxidime (68.61 %), Ciprofloxacin (69.96 %), Ofloxacin (70.85 %), Ertapenem (84.31%) Cefepime (85.20 %), Ticarcillin (89.24 %), Amikacin (90.59 %), Gentamicin (91.93 %) and Cefotaxime (93.72 %). The multiple antibiotic resistance index (MARI) ranges between 0.64 – 0.93. Result also revealed that biofilm producing *Pseudomonas* species from the water treatment plant harboured surface attachment genes namely: *pslA*, *pelA* and *algD*. PCR analysis showed that the identified organisms were *Pseudomonas aeruginosa* strain NA114 16S, *Pseudomonas tolaasii* strain ATCC 33618 16S, *Pseudomonas mendocina* strain Y20 16S, *Pseudomonas aeruginosa* strain F18 16S and *Pseudomonas mendocina* strain Y20 16S.

Keywords: Pseudomonas, Biofilm, Attachment Genes, Antibiotic Resistance, Water.

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1.0 Introduction

Bacteria in biofilm communities exhibit high tolerance to UV light exposure, higher rate of genetic exchange, enhanced production of secondary metabolites, multiple resistances to antibiotics and more efficient biodegrading abilities than their contemporary planktonic bacteria (Okafor *et al.*, 2022). Deterioration of the quality of drinking water caused by the formation of biofilm bacteria is a growing concern for most urban water supply agencies. In aquatic environments, microorganisms have the ability to adhere to solid surfaces and form biofilm (Okafor *et al.*, 2015). When biofilm is fully developed in the drinking water systems, bacterial re-growth in the distribution system may arise from the detachment and dissemination of biofilm bacteria. This enhances possible risk of infection in humans who may be the end users of the water (Onuoha *et al.*, 2016a).

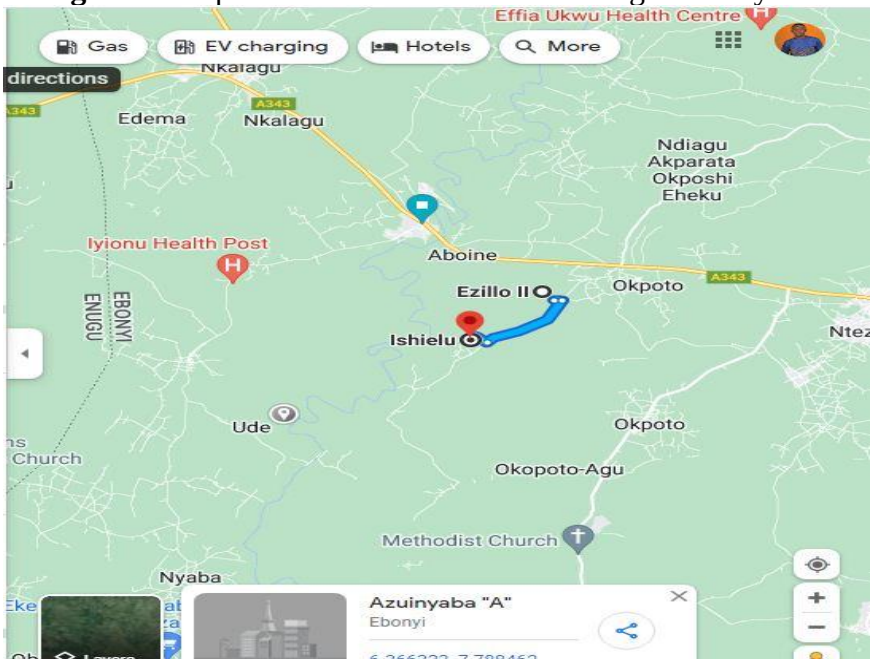
Bacteria in drinking water and other water distribution systems may grow in bulk water as biofilm attached to the walls of pipes (Ejikeugwu *et al.*, 2017), thus constituting a mixed community of bacteria which may enhance exchange of genetic components such as plasmids, integron, insertion elements and antibiotic resistance genes through horizontal transfer within the biolfilm matrix (Onuoha *et al.*, 2016b; Agah *et al.*, 2018; Okafor *et al.*, 2023). This may lead to the possible cause of antibiotic tolerance and resistance in bacteria (Ude *et al.*, 2021).

2.0 Methodology

2.1 Study Area

This research work was carried out on the water treatment plant located in Ezillo, Ishielu Local Government Area of Ebonyi State, Nigeria. Ishielu is a Local Government Area of Ebonyi State, Nigeria (figure 1). Its headquarters are in the town of Ezillo. Ishielu Local Government Area of Ebonyi State is bounded to the north by Benue State, to the south by Onicha Local Government and to the east by Ohaukwu and Ezza North Local Government and west by Enugu State. It has an area of 872 Km^2 and a population of 151, 048 at the 2006 National census (NPC, 2006). Ishielu LGA has a tropical climate with an average relative humidity of 75 % and may reach 80 % during rainy season. The area has annual rainfall between 2000mm and 2500mm and rain forest with atmospheric temperature between 21 ^{0}C and 31 ^{0} C. Two seasons are distinguishable in Ishielu LGA: a dry season (November to March) and a wet season (April and October). The Local Government area lie on longitude 7 ^{0}00E and 8 ^{0}00 and latitudes 4 ^{0}45 and 6 ^{0}17N. The inhabitant of this area are predominantly Igbo speaking people who are actively engaged in various activities such as trading, craft, agriculture, fish farming, government and public services. It has fertile soil with good soil composition for agricultural productivity which favors the production of cereal crops which are grown on a small holder plots usually in mixtures of at least two simultaneous crops such as yam, cassava, maize and other vegetables (Ugwumba and Isibor, 2014).

Figure 1. Map of Ishielu with red star showing the study area



2.2 Sample Collection and Analysis

Seventy (70) water samples comprising 19, 18, 16 and 17 were collected from flocculation bed, sedimentation bed, filtration bed and filtration discharge pipes respectively for bacteriological examination. The water samples were examined for bluish-green and yellowish-green pigmentation on Nutrient agar within 24 hours of collection and were further characterized. All suspected *Pseudomonas aeruginosa* isolates from the samples were separated and sub-cultured on cetrimide agar (OXOID, UK), whereas other *Pseudomonas* spp. were characterized and identified using a combination of conventional biochemical and microbiological analysis.

Biofilm formation was examined using the crystal violet (tube) binding assay method (Momba *et al.,* 2000; Iroha *et al.,* 2016; Herten *et al.,* 2017). Identified isolates were inoculated onto agar slant and kept in a bijou bottles and stored in refrigerator for further analysis.

2.3 Antibiotic Susceptibility Testing

The antibiotic resistance studies were done using Kirby-Bauer disc diffusion method. The antibiotic disks used for the study were Amoxicillin/Clavulanic acid (20/10 µg), Cefepime (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Ciprofloxacin, (5 µg), Gentamicin (10 µg), Ertapenem (10 µg), Imipenem (10 µg), Oxacillin (10 µg), Ofloxacin (5 µg), Amikacin (30 µg), Aztreonam (30 µg), Tobramycin (30µg) and Ticarcillin (75 µg) (Iroha *et al.,* 2010).

2.4 Molecular Analysis

Isolates obtained were further identified and characterized by PCR analysis using 16S rRNA primers.

Table 1. Oligonucleotide primers used

Target Gene	Primer Name	Primer Sequences (5' – 3')	Target	Expected Amplicon Size
Polysaccharide	**PslA-F**	**AAGATCAAGAAACGCGTGGAAT**	***psl*A**	**1700bp**
	PslA-R	**TGTAGAGGTCGAACCACACCG**	***psl*A**	
Pellicle	**PelA-F**	**CCTTCAGCCATCCGTTCTTCT**	***pelA***	**150bp**
	PelA-R	**TCGCGTACGAAGTCGACCTT**	***pelA***	
Alginate	**VIC-F**	**TTCCCTCGCAGAGAAAACATC**	***algD***	**500bp**
	VIC-R	**CCTGGTTGATCAGGTCGATCT**	***algD***	

Key: F = Forward primer, R = Reverse primer

Amplified 16S rRNA gene sequence was compared with sequences in NCBI sequence database using BLAST search program. The presence of surface attachment genes was determined using PCR analysis with specific primers (Table 1). DNA sequencing was carried out on amplified *pslA, pelA* and *algD* variants (Irie *et al.,* 2017; Moller *et al.,* 1996).

3.0 Results And Discussion

The distribution of biofilm producing *Pseudomonas* spp. isolates in water treatment reservoir is shown in Table 2. The frequency of distribution of *Pseudomonas* spp. from water treatment reservoir was high with a significant difference. Pseudomonas has become a predominant contaminant in most drinking water and its treatment facilities. They can out number other major bacteria contaminants such as *E. coli*, *Enterobacter* and *Klebsiella* in drinking water sources (Degayo *et al*., 2018). Their presence in drinking ware systems may pose possible risk for otitis externa, community-acquired pneumonias, and ventilator-associated pneumonias. These conditions are associated with lack of proper and periodic attention to water quality, thus making aquatic systems to be a good source of disease transmission. It is important to always investigate the quality of water meant for drinking, recreational and domestic purposes. Strict quality control measures should also be put in place to ensure the effective treatment of drinking water meant for public consumption (Okafor *et al.,* 2015b).

There is an increased level of occurrence of putative isolates of *Pseudomonas aeruginosa* in drinking water systems together with other bacteria across the globe (September *et al.,* 2007; Mulamattathil *et al.,* 2014; Onuoha *et al.,* 2016 a&b; Agah *et al.,* 2018; Okafor *et al.,* 2022; Onuoha *et al.,* 2023). This calls for a serious public health approach in ensuring that waterborne diseases are curtailed to a bearable minimum in the society. Our result revealed that *Pseudomonas* spp. isolated from Ezillo water treatment reservoir formed biolfilm communities with a statistical significance difference (P = .049) across different treatment beds in the plant (Table 2). The formation of biofilm by *Pseudomonas* spp. was not restricted to a particular site of the water treatment reservoir. This is an ugly development that demands urgent public health response in Nigeria because similar findings where *Pseudomonas* spp. isolated from public water supply displayed both high and low biofilm formation has been reported in Southwestern and Southeastern Nigeria respectively (Akaniro *et al.*, 2019; Okafor *et al.*, 2022).

Contrary reports that indicates *Pseudomonas* spp., seldom detected in drinking water systems by cultivation methods and that the organism is usually a minor fraction of the microbial community in the mature biofilm of water networks also exist (Loveday *et al.,* 2013). However, this does not suggest a negligent approach since the number of cells released from biofilm together with other cellular characteristics, and identity is a basic factor that can shape a drinking water microbiome (Irie *et al.,* 2017). The organism been reported to have a higher frequency of occurrence than other microorganisms associated with biofilm on pipe surfaces in the water distribution systems (Lais *et al.,* 2019). Poorly treated drinking water usually contains microbes that survive the treatment process or enter the distribution system through the pipe network. These microbes may attach to the pipe walls and become part of a biofilm matrix. These environmentally or disinfectant-stressed biofilm microbial communities usually re-grow under certain favourable conditions in the water system (Lais *et al.,* 2019).

Bacteria tend to become resistant to indiscriminate exposure to antimicrobial agents after previous exposure to extreme environmental conditions and antibiotic treatment. It is not unusual to find biofilm forming organisms showing high antibiotic resistance to commonly used antibiotics since they might have experienced both *in-vivo* exposures to the drugs during patients' treatment in the hospital with the antibiotics and *in-vitro* exposure to disinfectants used in cleaning an industrial or hospital environment. The dissemination of antibiotic-resistant bacteria from the hospital wastewater network (treated and untreated) to water bodies such as streams and rivers may also constitute potential risk of environmental contamination of water bodies (Shahid *et al.,* 2003).

Table 2: Distribution and Biofilm Production among *Pseudomonas* spp., Isolated from Water Treatment Plant.

Sample Site	No. of Samples Collected from Sites	Number Positive for *P. aeruginosa*	Number Positive for Biofilm Assay	Number Negative for Biofilm Assay
Flocculation	**19**	**17 (89.47%)**	**16 (28.07%)**	**1 (1.75%)**
Filtration discharge	**18**			**3 (5.26%)**
Filtration bed	**16**	**13 (81.25%)**	**5(8.77%)**	**8 (14.04%)**
Sedimentation bed	**17**	**15 (88.24%)**	**12 (66.66%)**	**9(15.79%)**
Total	**70**	**57 (81.43%)**	**45 (78.95%)**	**12 (21.05%)**
M±SD		**14.25±2.22**	**11.25±5.19**	**3.00±3.56**
Sig (2-tailed)		**0.001**	**0.023**	**0.196**

The resistance to most of the antibiotics used in this study by the test isolates could possibly stem from the fact that the minimum inhibitory concentration (MIC) of the antibiotics used were not adequate to inhibit the growth of organisms that exist in the biofilm matrices since microorganisms tend to survive and strive with a low MIC when they exist in a biofilm community (Ranita *et al.,* 2018). The high resistance pattern observed in this study is no doubt attributed to the ability of *Pseudomonas* isolates to form and exist in a biofilm matrix which increases their resistance to conventional antibiotics to about 1000-fold. Although other factors such as nature and structure of biofilm, intrinsic and acquired resistance, nutrient and oxygen availability to bacteria cells and metabolic state may also contribute to their tolerance of inhibitory agents at sub lethal concentrations (Okafor *et al*., 2022).

The test isolates showed a high degree of multiple drug resistance (MDR) to Imipenem and other carberpenems used in this study (Table 3). These antibiotics are presumably the last resort therapeutic agents against highly resistant bacteria in health care services. The carbapenems are very important antibiotics and plays significant role in the fight against bacterial infections because of their ability to resist the hydrolytic action of beta-lactamase enzyme. This makes them the broadest spectrum of antimicrobial agents among the several hundreds of known β-lactam drugs (Talukdar *et al.,* 2013). It is quite unfortunate and worrisome that bacterial pathogens that are resistant to these lives saving class of antibiotics has recently emerged in Nigeria (Akaniro *et al.,* 2019; Edet *et al.,* 2018). An increasing recovery of carbepenem-resistant Gram negative bacteria has been reported across the globe over the past two decades. These organisms' ability to produce carbepenemases and β-lactamases that can inactivate carbapenems and frequently used β-lactam antibiotics represents the major reason for their widespread (Patel and Bonormo, 2011; Ovia *et al.,* 2023).

Table 3: Antibiotics Susceptibility Pattern of *Pseudomonas* spp., Isolated Water Treatment Reservoir in Nigeria.

Antibiotics	Disc Potency (µg)	No Tested	No. Susceptible (%)	No. Resistant (%)
IPM	**10**	**56**	**34(60.71)**	**22(39.29)**
ETP	**10**	**56**	**9(16.07)**	**47(83.93)**
FEP	**30**	**56**	**8(14.29)**	**48(85.71)**
CAZ	**30**	**56**	**18(32.14)**	**38(67.86)**
CTX	**30**	**56**	**14(7.14)**	**42(92.86)**
AMC	**30**	**56**	**0 (0.00)**	**56(100.00)**
TOB	**10**	**56**	**0 (0.00)**	**56(100.00)**
TIC	**75**	**56**	**6(10.71)**	**50(89.29)**
OX	**10**	**56**	**0 (0.00)**	**56(100.00)**
ATM	**30**	**56**	**0 (0.00)**	**56(100.00)**
CN	**30**	**56**	**5(8.92)**	**51(91.93)**
AK	**30**	**56**	**6(10.71)**	**50(89.29)**
CIP	**5**	**56**	**17(30.36)**	**39(69.64)**
OFX	**5**	**56**	**16(28.57)**	**40(71.43)**

Key: IMP = Imipenem, ETP = Ertapenem, FEP = Cefepime, CAZ = Ceftazidime CTX = Cefotaxime, AMC = Amoxicillin, TOB = Tobramycin, TIC = Ticarcillin, OX = Oxacillin, ATM = Aztreonam, CN = Gentamicin, AK = Amikacin, CIP = Ciprofloxacin, OFX=Ofloxacin.

P. aeruginosa, *P. tolasii* and *P. mendocina* displaying multiple drug resistance (MDR) to different classes of antibiotics used in this study is an ugly scenario that needs a quick approach in order to checkmate the global spread of MDR among bacterial pathogens in water especially those with a nosocomial origin.

Table 4: Multiple Antibiotics Resistance Index (MARI) for *Pseudomonas* spp., Isolated from Water Treatment Reservoir.

Sample Site	MARI	Resistance Pattern
Flocculation bed	**0.85**	**CTX, AMC, TOB, TIC, OX, ETP, FEP, CAZ, CN, AK, ATM, CIP**
Sedimentation bed	**0.71**	**ETP, FEP, CTX, AMC, TOB, TIC, OX, ATM, CN, AK**
Filtration bed	**0.64**	**CTX, AMC, ETP, TOB, TIC, OX, ATM, CN, AK**
Filtration discharge	**0.93**	**ETP, FEP, CTX, AMC, TOB, TIC, OX, ATM, CN, AK, CIP, OFX, CAZ**
Mean±SD	**0.78±0.13**	
p-value	**0.002**	

The ability of *P. aeruginosa* to produce metallo-β-lactamase enzymes (MBL) which are major resistant factors in the organism over beta-lactam drugs and co-production of bla_{VIM} and bla_{OXA-48} and bla_{NDM} carbapenemase encoding genes, extended-spectrum β-lactamases (ESBLs) such as *PER*-1 and metallo-β-lactamases (*MBL*) such as *IMP*-29 among wild-type antibiotic-resistant and MDR has been reported across the globe (Slokevec *et al.,* 2012; Okafor *et al.,* 2023). Carbapenem resistant *P. aeruginosa* has become an emerging threat across the globe. This situation connotes an alarming threat of MBL production and necessitates a frequent routine screening of the organism for MBL, AmpC and ESBL production and also less toxic and cost effective chemothrapeutic agents for future imperative usage.

The isolates displayed multiple drug resistance to various antibiotics used in this study (Table 4). The lowest MARI index exceeded the acceptable limit (> 0.2) indicating indiscriminate exposure of the isolates to antibiotics (Okafor *et al.,* 2022). Multiple antibiotic resistances by bacterial isolates have become a leading global challenge that needs constant evaluation because of its huge negative impact to clinical medicine. In order to curtail the increasing trend of MDR that is on the increase in Nigeria, there is a need for the development and robust implementation of drug administration policies and effective monitoring to enforce strict adherence to such policies.

Table 5: Plasmid Curing Analysis of Pseudomonas spp., Isolated from Water Treatment Plants

Sampling Sites	No Positive for Plasmid (%)	No Negative for Plasmid (%)
Flocculation bed	**28 (71.79)**	**11 (28.21)**
Sedimentation bed	**19 (48.72)**	**20 (51.28)**
Filtration bed	**18 (46.15)**	**21 (53.85)**
Filtration discharge	**16 (42.11)**	**23 (58.97)**
Total	**39 (68.42)**	**18 (31.58)**
p-value	**0.068**	**0.058**

The result of our study on plasmid borne biofilm forming *Pseudomonas* spp isolated from water treatment plants is shown in Table 5. Further examination on the antimicrobial susceptibility revealed that their sensitivity to the antibiotics used was high compared to the uncured plasmid isolates (Table 6).

However, few isolates still maintained significant degree of resistance to some antibiotics such as aztreonam, amoxicillin/clavulanic acid, ceftazidime, ciprofloxacin. This suggests that the resistance to these antibiotics were not plasmid encoded but may be as a result of the presence of enzymes and other resistant genes.

The negative impact of acquired plasmid on bacteria host genome such as reduction in competitiveness is as a result of the ability of the plasmid to sequestrate the bacterial replication or expression machinery leading to inappropriate expression of newly acquired genes. This may lead to a complete loss of bacterial competitive fitness has been reported (Baltrus, 2013), signifying that plasmid encoded resistance poses a global health challenge.

The result of surface attachment exopolysaccharide genes in the test isolates is shown in Table 7. *Pseudomonas* spp. isolates analyzed in this study harboured surface attachment genes which are responsible for biofilm formation. The polysaccharide gene (*pslA*), pellicle gene (*pelA*) and alginate gene (*algD*) were identified. *Pseudomonas aeruginosa* strain NA114 16S, *Pseudomonas tolaasii* strain ATCC 33618 16S, *Pseudomonas mendocina* strain Y20 16S, *Pseudomonas aeruginosa* strain F18 16S, and *Pseudomonas mendocina* strain Y20 16S were characterized in this study.

Table 6: Antibiotic Susceptibility of Plasmid Cured Biofilm-forming Pseudomonas spp., Isolated from Water Treatment Plant to Selected Antibiotics.

Antibiotics Used	Disc Potency (µg)	No. Tested	No. (%) Susceptible	No. (%) Resistant
IPM	**10**	**32**	**28 (87.50)**	**4 (12.50)**
ATM	**30**	**32**	**8 (25.00)**	**24 (75.00)**
AMC	**30**	**32**	**20 (62.50)**	**12 (37.50)**
CAZ	**30**	**32**	**19 (59.38)**	**13 (40.63)**
CTX	**30**	**32**	**27 (84.38)**	**5 (15.63)**
CIP	**5**	**32**	**26 (81.25)**	**6 (18.75)**
Mean±SD			**21.33±7.53**	**10.67±7.53**
p-value			**0.001**	**0.018**

KEY: IMP = Imipenem, CAZ = Ceftazidime CTX = Cefotaxime,
ATM = Aztreonam, AMC = Amoxicillin/ Clavulanic acid, CIP = Ciprofloxacin.

Biofilm bacteria is well known to produce one or more complex polysaccharides that account for the semi-solid domed cluster linked with water molecules that stabilizes and reinforce the structure of the biofilm, mediate cell-cell communication (quorum sensing using *N*-acyl homoserine lactone signal molecules) and cell-surface attachment appendages such as pellicle, pili and flagella. These factors selectively allow the passage of materials and shield the bacteria in the biofilm matrix from biocides and antimicrobial agents (Irie *et al.*, 2017).

The presence of alginate in *P. aeruginosa* is crucial to the organism pathogenesis and biofilm production. The presence of *algD* genes in *P. aeruginosa* and the identification of its key role in alginate synthesis and mucoid formation in *P. aeruginosa* have been reported (Sultan *et al.*, 2021). *P. aeruginosa* capable of forming biofilm under stress conditions has also been reported (Kekeç, *et al.*, 2016). A Gene expression study has identified the alginate gene to be among six genes responsible for strain and stress response and biofilm trigger mechanism in opportunistic and pathogenic *P. aeruginosa* PAO1 (Kekeç, *et al.*, 2016). Alginate has been reported as one of the major exopolysaccharide of *Pseudomonas aeruginosa* biofilm matrix, with minimal regard to the different function polysaccharides carries out in biofilm formation. Although alginate is not involved in the initiation of biofilm formation, it plays very significant role in the further development and maturation of the biofilm in some strain of *P. aeruginosa* (Sultan *et al.*, 2021). At least three exoplysaccharides (alginate, *psl* and *pel*) responsible

for the formation of biofilm in bacteria has been identified (Irie *et al.,* 2017). These exopolysaccharides plays extensive role in the biofilm matrix (Sultan *et al.,* 2021).

The lack of *psl* enhances the production of *pel* whereas the absence of *pel* stimulates the production of alginate. Previous work has also described the role and specific interrelated function of biofilm forming genes of which the genes for alginate and *psl* and *pel* has been extensively reported (Ghafoor *et al.,* 2011) among different strains of *Pseudomonas* spp. including *Pseudomonas mandelii, Pseudomonas aeruginosa* and *Pseudomonas fluorescens*.

Table 7: Distribution of Surface Attachment Genes among *Pseudomonas* spp., from Water Samples

Isolate Code	Pairwise Identity (%)	Identified Organism	Gene Detected	Amplicon Size (bp)
$F_{10}P_2$	**76.10**	***Pseudomonas aeruginosa* strain NA114 16S**	***pelA, algD***	**120, 550**
$F_{17}P_2$	**83.30**	***Pseudomonas tolaasii* strain ATCC 33618 16S**	***pelA***	**120**
$Fd_{48}P_2$	**91.00**	***Pseudomonas mendocina* strain Y20 16S**	***pelA, algD***	**120, 550,1750**
$F_{12}P_2$	**83.30**	***Pseudomonas aeruginosa* strain F18 16S**	***pelA, algD, pslA***	**120, 550,1750**
F_{19}	**90.50**	***Pseudomonas mendocina* strain Y20 16S**	***pelA, algD,***	**120, 550**

The presence and involvement of *psl* in the initial attachment of cells and the role of *pel* in the maturation of the biofilm matrix and entrapment of more cells into the biofilm matrix has been examined in previous studies (Colvin *et al.,* 2011; Vásquez-Poncea *et al.,* 2017). Our findings on the presence of these genes that are responsible for the production of the exopolysaccharides used for attachment and maintenance of bacteria biofilm are consistent with these studies. Biofilm matrices contribute significantly to antimicrobial resistance in bacteria posing a challenge that needs to be curbed in order to curtail the spread of resistance strains across the globe.

4.0 Conclusion

This study has shown that water samples obtained from water treatment plants harboured *Pseudomonas* species that were biofilm producers. Biofilm forming potentials present in the isolates makes them difficult to eradicate from the drinking water treatment reservoir thus impeding the conventional treatment processes. Treatment of drinking water meant for public consumption should be upgraded at the reservoir plant in order to improve the quality of drinking processed at the treatment plant.

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