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PLASMID CURING ANALYSIS OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM DIFFERENT DRINKING WATER SOURCES IN AFIKPO NORTH L.G.A, EBONYI STATE, NIGERIA

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Abstract

This study investigates plasmid curing analysis on Pseudomonas aeruginosa isolated from Wowoo River and Otu-eke (Ndibe Beach) in Afikpo North LGA. Total active bacterial count ranged from 2.0 x 10^5 to 3.0 x 10^5 cfu/ml. Pseudomonas aeruginosa was presence 30% of Otu-eke River (Ndibe Beach) water samples and 35% of Wowoo river water sample. The bacteria were entirely susceptible to ceftazidime and Aztreonam. The resistance rate is in this variation: High resistance to amoxicillin (83.33%), and moderately resistance to imipenem (66.66%). Multiple Antibiotic Resistance Index: 0.66 for Wowoo stream and 0.44 for Otu-eke River (Ndibe beach). A total of 75% of isolated P. aeruginosa harboured plasmids. Plasmid-borne Pseudomonas aeruginosa found in both river drinking water in Afikpo is a thing of concern as it's spreading resistance in clinical isolates. This study concludes that Otu-eke (Ndibe Beach) and Woowo river, as a source of water for human use, poses a significant health risk due to the high presence of coliform bacteria, including Pseudomonas aeruginosa, which demonstrated multiple antibiotic resistance. The resistance genes were found to be both plasmid and chromosomally borne, highlighting the need for caution when using both Otu-eke (Ndibe Beach) and Woowo river water for human consumption or other purposes.

Keywords: Water, Pseudomonas aeruginosa, antimicrobial susceptibility, Plasmid curing, Afikpo.

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium that is opportunistic and ubiquitous in the environment, found in animals, soil, plants, water, and sewage (Frank, 2012). It is a major human pathogen that causes severe complications in immunocompromised patients and is implicated in nosocomial infections, including bacteraemia, burn infections, cystic fibrosis, pneumonia, and urinary tract infections (Islam *et al., 2020*). The bacterium has developed resistance to many antibiotics, making treatment challenging (Arora *et al., 2011*; Chika *et al., 2017*), and has been included in the World Health Organization's list of priority pathogens requiring new antibiotics. *P. aeruginosa*'s ability to acquire new resistance genes is enhanced by its dispersion in aquatic environments and horizontal gene transfer (Mesaros *et al., 2007*). Previous studies in Nigeria have focused on clinical environments and industrial wastewater effluents (Adesoji *et al., 2016*).

This study aims to isolate and analyse the plasmid curing of *P. aeruginosa* isolated from Wowoo River and Otu-eke (Ndibe Beach) sources in Afikpo North LGA, to identify plasmid encoded strains among the test isolates from the water sources, to screen for antibiotic resistance among plasmid encoded isolates from the

water sources. This study also screened the effects of plasmid mediated resistance among tested isolates from the water sources. In this study's findings, it can help control the spread of *P. aeruginosa* in water sources, maintain healthy life and safe drinking water, and guide treatment of *P. aeruginosa* infections in cases of antibiotic resistance.

The study was carried out in Ndibe beach and Woowo River both Afikpo North L.G.A in Ebonyi State South East Nigeria. Ndibe beach Afikpo North L.G.A in Ebonyi State South East Nigeria, geographically it is located on latitude 60N and longitude 80E of the Greenwich meridian with the estimated population of 156,611 according to the Nigeria 2006 census. Woowo River is a natural freshwater body located along Unwana Road in Afikpo North Local Government Area of Ebonyi State, South-East Nigeria. The river lies within the Unwana axis, an area that forms part of the larger Afikpo region known for its scenic landscapes, rich cultural heritage, and proximity to the Cross River basin. Afikpo North is situated on latitude approximately 6°00'N and longitude 8°00'E of the Greenwich Meridian. The LGA serves as one of the major population and commercial centres in Ebonyi State, with its administrative headquarters in Ehugbo (Afikpo town). Unwana and the surrounding communities, including the Wowoo River area, are primarily rural settlements where water bodies like the Wowoo River and Ndibe Beach play significant roles in domestic, recreational, and agricultural activities.

Materials and Methods (Methodology)

Sample Collection

A total of 40 water samples were collected, 20 from each of Wowoo river and Otu-eke (Ndibe Beach) drinking water sources in Afikpo North LGA, Ebonyi State, Nigeria. The samples were collected in sterile Bijou bottles and transported to the Microbiology Laboratory Unit of Akanu Ibiam Federal Polytechnic, Unwana, for bacteriological analysis.

Isolation of Bacteria

Ten-fold serial dilutions of each water sample were carried out, and 1ml of 10^5 dilution was plated in duplicates on cetrimide agar plates using the pour plate technique (Prescott et al., 2005). The plates were incubated at 37°C for 24 hours. Bacterial colonies were counted, and colonies differing in size, shape, and colour were selected and subcultured on nutrient agar.

Identification and Characterization of Isolates

The isolates were identified based on their colonial appearance, including colour, shape, form, and consistency (Cheesbrough, 2000). The isolates were further characterized using biochemical tests.

Standardization of bacterial inoculum

A loop full of test bacterial isolates were inoculated on nutrient broth and incubated for 24 hours.0.2ml from the 24 hours broth culture of the bacteria from each sample media were dispensed into 20ml sterile nutrient broth and incubated for 3 to 5 hours to standardize the culture to 0.5 McFarland standards (106CFU/ml) before use according to method described by Clinical Laboratory Standards Institute (CLSI) (2014).

Preparation of McFarland Turbidity Standard

0.5 McFarland turbidity standard is the mixture of 1% barium chloride ($BaCl_2.2H_2O$) and 1% sulphuric acid solution (H_2SO_4). After incubation, a culture was obtained and it was used to prepare the inoculums. A sterile wire loop was used to collect and transfer the colonies into a test tube containing 5mls of normal saline and emulsified. The turbidity of the suspension was checked by placing it against a plain paper with written words to see if the words appear blurred. The suspension appear blur to show that it was up to 0.5

McFarland turbidity standard. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion technique on Mueller Hinton agar plates (CLSI, 2014). The isolates were tested against various antibiotics, including Imipenem, Ceftazidime, Augmentin, Amikacin, Gentamicin, Aztreonam, Cefoxitin, and Ofloxacin.

Preparation of Inoculum Bed / Inoculation

After preparing the inoculum suspension, a sterile swab stick was dipped in to the suspension, and the excess was pressed against the sides of the test tube above the suspension level. The swab was streaked to cover the entire surface of the medium by rotating the plate approximately 60^{o} to ensure even distribution. The petri dish was kept in a place and allowed to dry for few minutes for the surface of the Muller Hinton agar to dry (Okeke and Amadi, 2001; Cheesbrough, 2000).

Antimicrobial Susceptibility Testing

Antibiotic susceptibility test was performed on the isolates using Kirby Bauer's disc diffusion technique on freshly prepared Mueller Hinton's agar plates. Using swab stick, the suspension of each bacterial isolates, and equivalent to 0.5 McFarland Standard wad aseptically seeded into Mueller Hinton agar plates respectively. The antibiotic disc containing Imipenem (10µg), Ceftazidime (30mg), Augmentin (30µg), Amikacin (30)Gentamicin (10µg), Aztreonam (10), Cefoxitin (30µg),and Ofloxacin (5µg) were aseptically placed on the surface of the molten Mueller Hinton agar plate and allowed for 30 minutes to pre-diffuse for each of the sample. These were incubated for18-24hours at 23°C after which the diameter of zones of Inhibitions will be recorded.

Determination of Multiple Antibiotic Resistance Index

The multiple antibiotic resistance index was determined using the formula MAR = x/y, where x is the number of antibiotics to which the test isolate displayed resistance, and y is the total number of antibiotics to which the test organism was evaluated for sensitivity (Olayinka et al., 2009).

Plasmid Curing of *Pseudomonas aeruginosa* Isolates

Fifty micro litres of acridine orange (10ml) were added to 5ml of Nutrient broth. 2-5 colonies (loop-full) of *Pseudomonas aeruginosa* was inoculated to the LB broth having acridine orange (50nm/ml) respectively. It was then incubated for 24hours in a shaker incubator. After the 24hours of incubation, the culture was swabbed into Mueller Hinton agar plates for each sample, then appropriate antibiotics disc was aseptically introduced into the plates, ensuring that the disc made appropriate contact with the surface of the agar. These will be incubated for 24hours at 23°C, after which the plates were examined

Post-Curing Sensitivity Testing

The plasmid cured isolates of *Pseudomonas aeruginosa* was tested against those antibiotics to which they were previously resistant. Post curing sensitivity tests were performed on the isolates using Kirby Bauer's agar disc diffusion technique as described by Clinical Laboratory Standard Institute (CLSI) using overnight broth culture. Mueller-Hinton agar plates were prepared according to manufacturer's specification. The isolates were sub-cultured onto freshly prepared nutrient agar plates for each sample and incubated at 23°C for 18 hours. A 1ml suspension of each bacterial isolates from each sample, equivalent to McFarland standards will be aseptically seeded into Mueller-Hinton agar plate respectively. These were allowed to stand for 1 hour to solidify. The antibiotic paper disc containing Imipenem (10µg), Augmentin (30µg), Amikacin (30µg), Ceftazidime (30µg),Gentamicin (10µg), Cefoxitin (30µg), Aztreonam (10µg) and Ofloxacin (5µg), were aseptically placed on the surface of the molten Mueller Hinton agar and allowed for 30 minutes to pre-diffuse. These were incubated for 18-24 hours at 23°C after which the diameters of zone of inhibition were measured using meter rule in mm and zones were compared with standard antibiotics

chart. The methodology used in this study followed standard microbiological procedures and guidelines for antimicrobial susceptibility testing and plasmid curing.

Results

The results of the study are presented in the tables below:

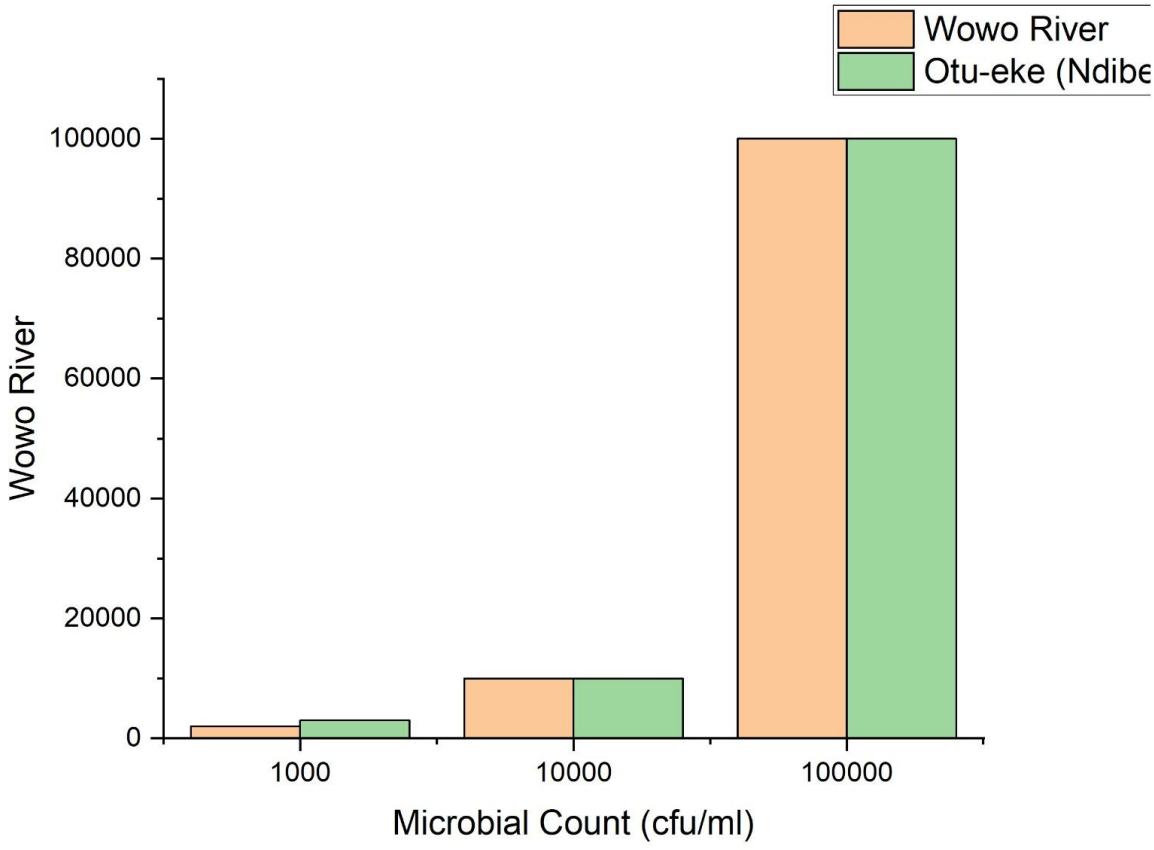
Microbial Content of Water Samples

The total bacterial count of *P. aeruginosa* in the water samples from Wowoo River and Otu-Eke (Ndibe Beach) exceeded the recommended limits of 100 cfu/ml set by NSDWQ (2007). The bacterial count ranged from 2.0 x 10^3 cfu/ml to 1.0 x 10^5 cfu/ml in both river sources (Table 1).

Table 1: Microbial Content of Water Samples

S/N	Microbial Count (cfu/ml)	Wowoo River	Otu-eke (Ndibe Beach)	Standard Recommended Limits
1	Total Bacterial X 10^3	2.0 x 10^3 (cfu/ml)	3.0 x 10^3 (cfu/ml)	100
2	Total Bacterial X 10^4	1.0 x 10^4 (cfu/ml)	1.0 x 10^4 (cfu/ml)	100
3	Total Bacterial X 10^5	1.0 x 10^5 (cfu/ml)	1.0 x 10^5 (cfu/ml)	100

Fig. 1 Microbial content Wowoo and Otu-eke



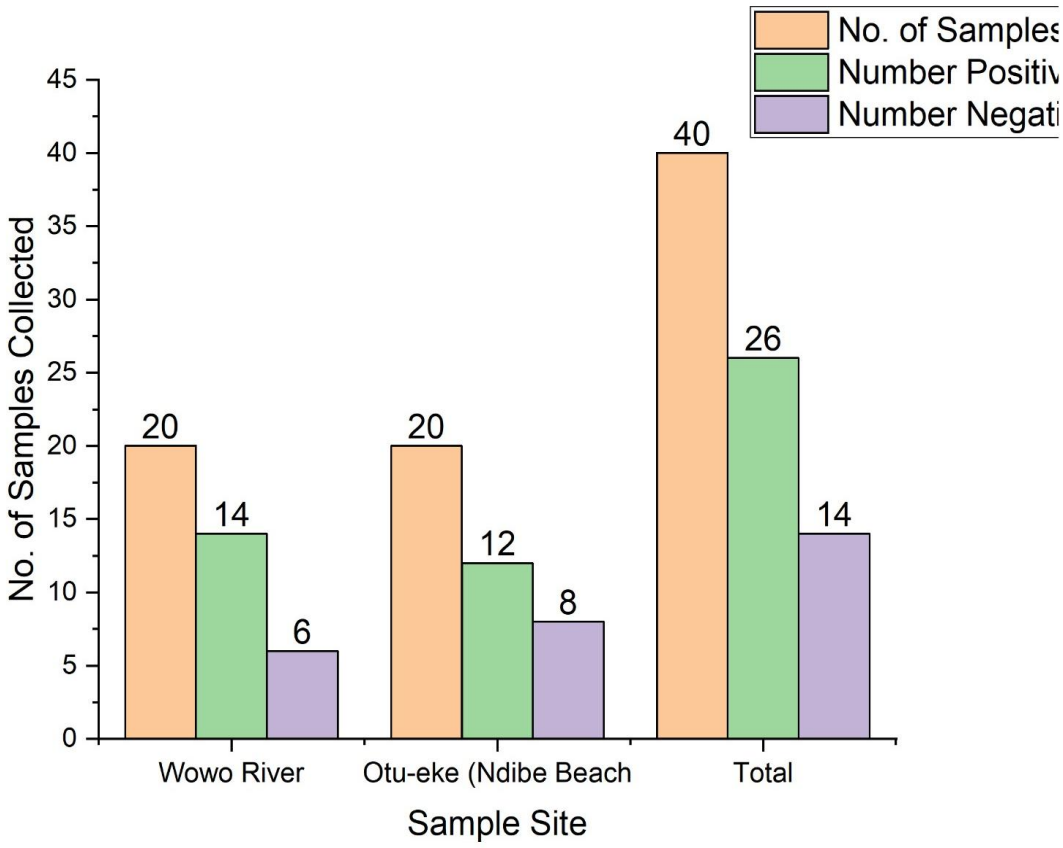
Distribution of Pseudomonas aeruginosa

Out of 40 samples collected, a total of 26 (65%) were positive for *P. aeruginosa*, with 14 (35%) samples from Wowoo River and 12 (30%) samples from Otu-eke (Ndibe Beach) respectively (Table 2).

Table 2: Distribution of *Pseudomonas aeruginosa*

Sample Site	No. of Samples Collected	Number Positive for P. aeruginosa	Number Negative for P. aeruginosa
Wowoo River	20	14 (35%)	6 (15%)
Otu-eke (Ndibe Beach	20	12 (30%)	8 (20%)
Total	40	26 (65%)	14 (35%)

Fig. 2 Distribution of *Psedomonas Aeruginosa*



Morphological and Biochemical Identification

The isolates showed characteristic colonial morphology, including blue-green, yellowish-green, and milky colours, and were identified as *P. aeruginosa* based on biochemical tests (Table 3).

Table 3: Morphological and Biochemical Identification

Colour	Translucency	Colony Edge	Shape	Size	Elevation	Consistency	Margin	Oxidase	Catalase	Gram Reaction	Citrate
Blue-green	Opaque	Entire	Flat	Moderate	Umbonate	Mucoid	Irregular	+	+	-	+

Antibiotic Susceptibility Pattern

The isolates showed varying levels of susceptibility to different antibiotics, with 100% susceptibility to Ceftazidime and Aztreonam, and high resistance to Amoxicillin and Cefoxitin (Table 4).

Table 4: Antibiotic Susceptibility Pattern

Antibiotics	Disc Potency (µg)	No Tested	No. Susceptible (%)	No. Resistant (%)
IMP	10	24	8 (33.33)	16 (66.66)
CAZ	30	24	24 (100)	0 (0.00)
AMC	30	24	4 (16.66)	20 (83.33)
ATM	30	24	24 (100)	0 (0.00)

KEY

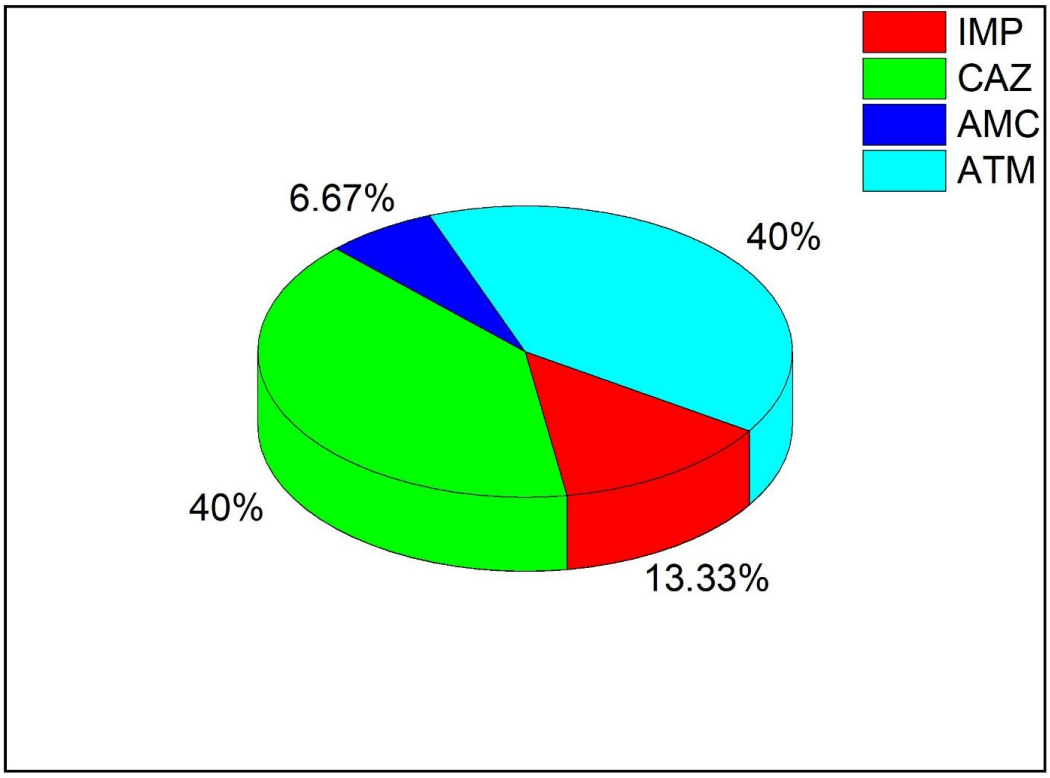
IMP = Imipenem

CAZ = Ceftazidime

AMC = Amoxicillin

ATM = Aztreonam

Fig. 3 Subtibility pattern of P. Aeruginosa to different Antibiotics



Multiple Antibiotic Resistance Index

According to European Centre for Disease Prevention and Control [ECDC] and the U.S. Centres for Disease Control and Prevention [CDC]) the multiple antibiotic resistance index (MARI) ranged from 0.44 to 0.66, indicating a high level of resistance to multiple antibiotics (Table 5).

Table 5: Multiple Antibiotic Resistance Index

Sample Site	MARI	Resistance Pattern
Otu-eke (Ndibe Beach)	0.66	AMC, OX, CN, AK, IMP, FOX
Wowoo River	0.44	AMC, OX, IMP, FOX

Plasmid Curing Analysis

The plasmid curing analysis showed that 75% of the isolates had plasmids that were cured, resulting in increased susceptibility to antibiotics (Table 6).

Table 6: Plasmid Curing Analysis of *Pseudomonas* spp., **Isolated from selected river sources in Afikpo North LGA**

Sampling Site	No Tested	No Positive for Plasmid (%)	No Negative for Plasmid (%)
Unwana River	8	5 (62.50)	3 (37.50)
Wowoo River	4	4 (100)	0 (0.00)
Total	**12**	**9 (75.00)**	**3 (25.00)**

This table above shows plasmid curing analysis of *Pseudomonas spp* which showed number of plasmid cured isolates of 5 (62.50 %) out of eight isolates from Unwana beach samples while 3 (37.50 %) were not plasmid cured and all four 4 (100 %) isolates from Wowoo River samples showed positive to plasmid.

Table 7: Antibiotic Susceptibility of Plasmid Cured *Pseudomonas* aeruginosa **Isolated from selected river sources in Afikpo North LGA**

Antibiotics	Disc Potency (µg)	No Tested	No. Susceptible (%)	No. Resistant (%)
CAZ	30	8	8(100)	0(0.00)
AMC	30	8	4 (50.00)	4 (50.00)
OX	10	8	0 (0.00)	8(100)
ATM	30	8	8 (100)	0(0.00)
CN	30	8	8 (100)	0(0.00)
AK	30	8	8 (100)	0(0.00)
OFX	5	8	8 (100)	0(0.00)

Key: CAZ = Ceftazidime, AMC = Amoxicillin/Clavulanic acid, OX = Oxacillin, ATM = Aztreonam, CN = Gentamicin, AK = Amikacin, OFX=Ofloxacin.

After plasmid curing analysis of the *Pseudomonas aeruginosa*, the antibiotics susceptibility pattern of *Pseudomonas aeruginosa* varied that all *Pseudomonas spp* were susceptible to Ceftazidime (30µg), Aztreonam (30µg), Gentamicin (30µg), Ofloxacin (5µg) and Amikacin (30µg), 50% were susceptibility to Amoxicillin (30µg)and none were susceptibility to Oxacillin (10µg) (table7). Thus, this showed that the cause of resistant was plasmid mediated as the number of susceptible isolates increased.

Fig. 4 Plasmid Curing Analysis

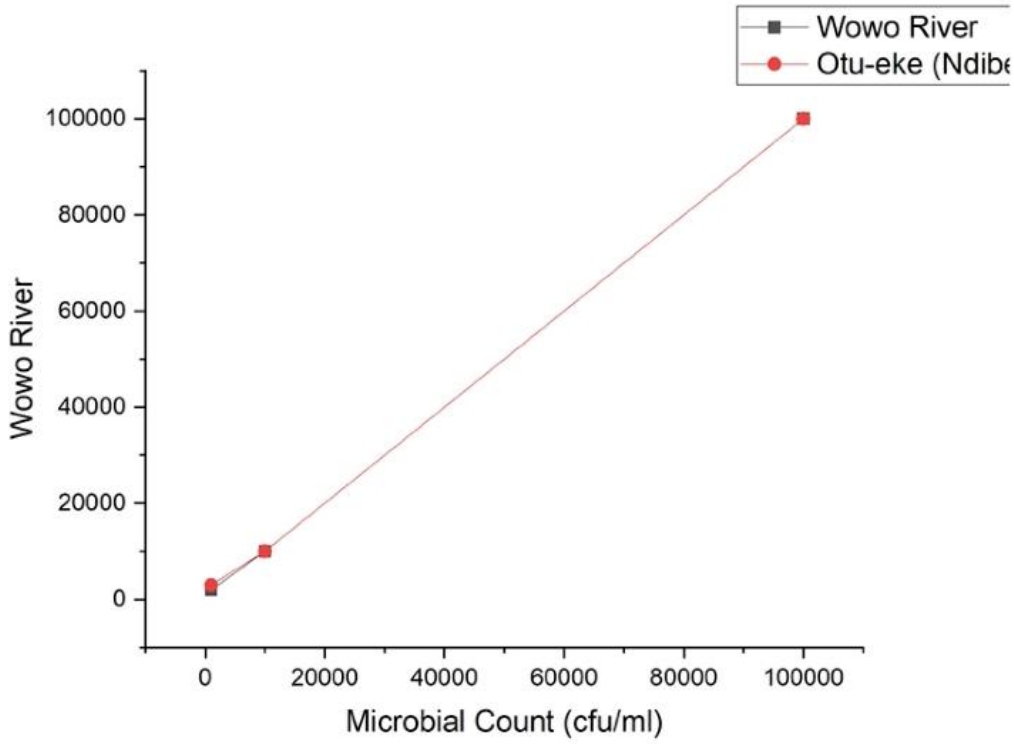
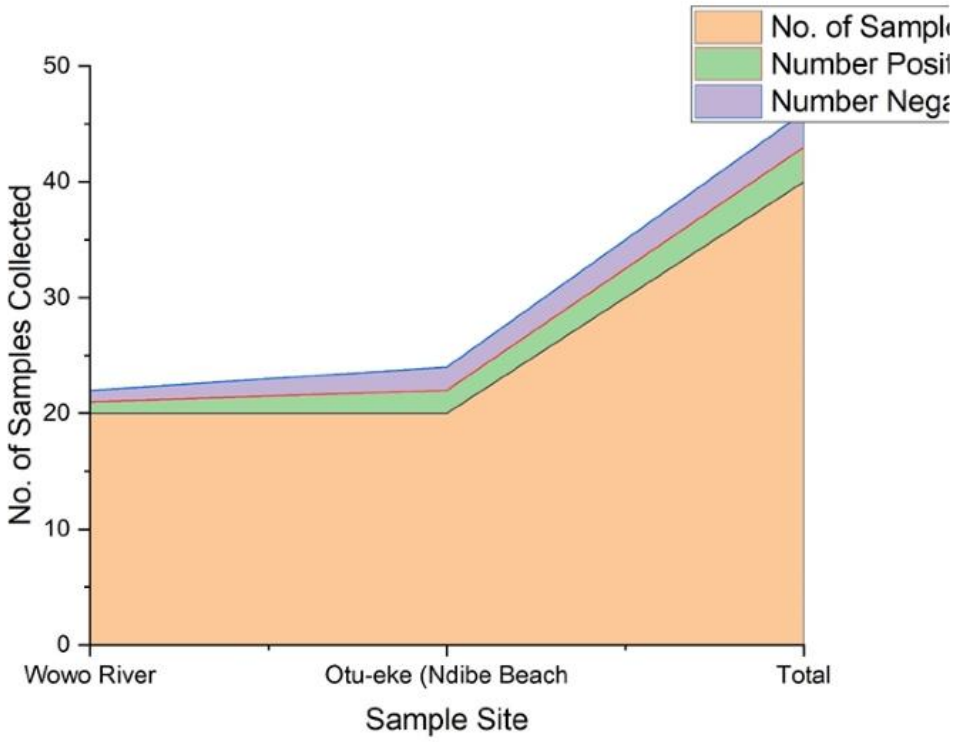


Fig. 5 Negative and positive of Plasmid Curing



Discussion

This study investigated the presence and plasmid-mediated antibiotic resistance of *Pseudomonas aeruginosa* isolated from Wowoo River and Otu-eke (Ndibe Beach) in Afikpo North Local Government Area of Ebonyi State, Nigeria. The findings revealed a high occurrence of *P. aeruginosa* in both water sources, indicating significant bacterial contamination and potential public health risks to individuals who rely on these rivers for drinking, domestic, or agricultural purposes. Similar observations have been reported by Adesoji et al. (2016), who detected multidrug-resistant *P. aeruginosa* in wastewater effluents from northern Nigeria, highlighting the role of aquatic environments as reservoirs of resistant bacteria.

The total viable bacterial count (TVBC) of the river samples ranged from 2.0×10^3 to 1.0×10^5 cfu/ml, which far exceeds the recommended limit of 100 cfu/ml set by WHO (2004) and the Nigerian Standard for Drinking Water Quality (NSDWQ, 2007). This high bacterial load suggests significant microbial pollution, possibly from domestic sewage, agricultural runoff, or other anthropogenic sources. Comparable findings were reported by Igbinosa et al. (2012), who observed elevated bacterial counts in contaminated surface waters in southern Nigeria, emphasizing the poor sanitation practices in many rural communities.

The antibiotic susceptibility results revealed that *P. aeruginosa* isolates were resistant to several commonly used antibiotics, including amoxicillin and cefoxitin, while showing complete susceptibility to ceftazidime and aztreonam. The calculated Multiple Antibiotic Resistance Index (MARI) values of 0.44 and 0.66 for Wowoo River and Otu-eke (Ndibe Beach), respectively, are well above the acceptable limit of 0.2. According to Krumperman (1983), MARI values above 0.2 indicate that bacteria originate from environments with frequent antibiotic exposure, such as hospitals, animal farms, or sewage-contaminated sites. These findings align with those of Chika et al. (2017), who reported similar resistance trends among *P. aeruginosa* isolates from hospital environments in southeastern Nigeria.

Plasmid curing analysis showed that 75% of the isolates lost their resistance traits after plasmid elimination, confirming that the observed antibiotic resistance was largely plasmid-borne. This finding supports previous studies (Mesaros et al., 2007; Islam et al., 2020) which established that *P. aeruginosa* can harbour resistance genes on transferable plasmids, allowing rapid dissemination of resistance through horizontal gene transfer. The persistence of resistance even after curing in some isolates suggests that additional chromosomal mechanisms, such as efflux pumps or enzyme-mediated resistance, may also be involved.

The detection of plasmid-mediated multidrug-resistant *P. aeruginosa* in these surface waters underscores a serious environmental and public health threat. Untreated or poorly treated river water used for domestic purposes could serve as a vector for resistant pathogens, leading to infections that are difficult to treat. This situation reflects the growing global concern over antimicrobial resistance in environmental reservoirs (WHO, 2023).

In summary, this study demonstrates that *P. aeruginosa* isolates from Wowoo River and Otu-eke (Ndibe Beach) possess significant multidrug resistance, much of which is plasmid-mediated. These findings emphasize the urgent need for continuous monitoring of environmental water sources, enforcement of proper waste disposal practices, and public health interventions to mitigate the spread of resistant bacteria in the community.

Conclusion

This study investigated the plasmid-mediated antibiotic resistance of *Pseudomonas aeruginosa* isolated from Wowoo River and Otu-eke (Ndibe Beach) in Afikpo North Local Government Area, Ebonyi State. The findings revealed that the bacterial counts in both water sources exceeded the

National Standards for Drinking Water Quality (NSDWQ, 2007) limits, indicating microbial contamination. A high proportion of the isolates exhibited multiple antibiotic resistance, with some resistance traits found to be plasmid-borne. This demonstrates that plasmids play a major role in the dissemination of antibiotic resistance among environmental bacteria.

The study emphasizes the importance of continuous monitoring of water sources for microbial contamination and antibiotic resistance. Ensuring safe and potable water supply through effective treatment and proper environmental management is crucial to safeguarding public health and preventing the spread of resistant pathogens.

Recommendations

Based on the key findings of this study, which analysed *Pseudomonas aeruginosa* isolated from Wowoo River and Otu-eke (Ndibe Beach) in Afikpo North L.G.A, Ebonyi State, the following recommendations are made:

1. The water from Wowoo River and Otu-eke (Ndibe Beach) should not be used directly for drinking or domestic purposes without adequate treatment. Proper disinfection methods such as boiling, chlorination, or filtration should be practiced at the household level to reduce microbial contamination and health risks.
2. Government and local authorities should prioritize the provision of safe and treated water to communities within Afikpo North L.G.A to minimize dependence on potentially contaminated surface water sources like Wowoo River and Otu-eke (Ndibe Beach).
3. There should be strict enforcement of environmental regulations to prevent indiscriminate dumping of wastes and untreated sewage into natural water bodies, as these contribute to the spread of antibiotic-resistant bacteria, including *Pseudomonas aeruginosa*.
4. Periodic microbiological monitoring of Wowoo River, Otu-eke (Ndibe Beach), and other water sources in the area should be carried out to assess microbial quality and detect emerging patterns of antibiotic resistance early.
5. Public health education campaigns should be conducted to create awareness among residents on the dangers of using untreated river water for drinking and domestic purposes, and to encourage community participation in environmental sanitation.
6. Wastewater and sewage generated within the community should be properly treated before discharge to prevent contamination of surrounding water sources and reduce the dissemination of resistance genes in the environment.

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