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Phenotypic Detection of Extended-Spectrum β-Lactamases (ESBLs), AmpC β-Lactamases, and Metallo-β-Lactamases (MBLs) in *Pseudomonas aeruginosa* Isolated from Drinking Water Sources at Iyi-Oka Spring and Unwana Beach, Afikpo North Local Government Area, Ebonyi State, Nigeria

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Abstract

This study phenotypically detected extended-spectrum beta-lactamase (ESBL), AmpC beta-lactamase, and metallo-beta-lactamase (MBL) production in *Pseudomonas aeruginosa* isolated from Unwana Beach and Iyi-Oka river water in Afikpo North LGA, Ebonyi State, Nigeria. Twenty water samples, ten from each site, were collected and analyzed using standard microbiological and biochemical methods, while antimicrobial susceptibility testing and enzyme detection were performed by Kirby-Bauer disc diffusion in line with CLSI guidance. The total bacterial load was high in both sources, with counts ranging from 3.4×1053.4 \times 10^53.4×105 to 21.1×10821.1 \times 10^821.1×108 CFU/ml in Iyi-Oka samples, while Unwana samples were reported as too numerous to count at the tested dilutions. *P. aeruginosa* was recovered from 17 of 20 samples (85%), with 70% positivity in Unwana River and 100% in Iyi-Oka River. The isolates showed greatest resistance to amoxicillin/clavulanic acid, oxacillin, cefoxitin, imipenem, aztreonam, and ceftazidime, but remained susceptible to gentamicin, amikacin, and ofloxacin. AmpC and ESBL were detected in both river isolates, whereas MBL was not detected. These findings suggest contamination of the water sources with multidrug-resistant *P. aeruginosa* and highlight a public health risk associated with untreated drinking water.

Keywords: *Pseudomonas aeruginosa;* Antibiotic resistance; River water; AmpC β-lactamase; Extended-spectrum β-lactamase (ESBL); Drinking water quality

1. Introduction

Pseudomonas aeruginosa is a Gram-negative, motile bacterium commonly found in soil, water, sewage, and other moist environments. It is an important opportunistic pathogen and is often implicated in hospital-associated infections, particularly among immunocompromised patients, burn victims, and individuals with chronic wounds or respiratory disease. Its clinical importance is amplified by its intrinsic resistance to several antimicrobial agents and its ability to acquire additional resistance mechanisms. [8,9] Previous investigations conducted in Afikpo North Local Government Area and other parts of Ebonyi State also demonstrated poor microbiological quality of surface water, high

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heterotrophic bacterial counts, and contamination with antibiotic-resistant bacteria, highlighting the persistent public health risks associated with untreated water sources and the need for continuous environmental surveillance. [13,17]

Beta-lactamase production is one of the most important mechanisms by which *P. aeruginosa* evades beta-lactam antibiotics. [2] ESBLs hydrolyze extended-spectrum cephalosporins [1], while AmpC enzymes confer resistance to cephamycins and many penicillins [3,4]; both may be encoded chromosomally or carried on mobile genetic elements, increasing their potential for spread. [4] MBLs are also clinically significant because they can inactivate carbapenems, which are often reserved as last-line drugs for severe infections. [7]

Water sources can serve as reservoirs and transmission pathways for resistant bacteria, especially where sanitation and treatment are inadequate. Previous studies have reported *Pseudomonas* and other bacteria from surface and drinking water sources, reinforcing the need for routine monitoring. [8,9] Previous investigations conducted in Afikpo North Local Government Area and other parts of Ebonyi State also demonstrated poor microbiological quality of surface water, high heterotrophic bacterial counts, and contamination with antibiotic-resistant bacteria, highlighting the persistent public health risks associated with untreated water sources and the need for continuous environmental surveillance. [13,17]

The aim of this study was therefore to phenotypically detect AmpC, ESBL, and MBL production in *P. aeruginosa* isolated from Unwana Beach and Iyi-Oka rivers in Afikpo North LGA, Ebonyi State.

2. Materials and Methods

2.1. Study Area and Sample Collection

Twenty (20) water samples were collected from two river sources in Afikpo North Local Government Area, Ebonyi State, Nigeria: ten (10) from Unwana Beach River and ten (10) from Iyi-Oka River. Samples were collected aseptically in sterile Bijou bottles, properly labelled, and transported immediately to the Microbiology Laboratory Unit, Department of Science Laboratory Technology, Akanu Ibiam Federal Polytechnic, Unwana, for bacteriological analysis.

2.2. Culture Media and Sterilization

All glass wares were sterilized by autoclaving at 121°C for 15 minutes. Three culture media were employed: Nutrient Agar (28 g/L), Centrimide Agar (45 g/L with 10 ml glycerol/L), and Mueller Hinton Agar (38 g/L), each prepared according to the manufacturer's specifications and autoclaved at 121°C for 15 minutes. Prepared plates were stored at 3–8°C until use.

2.3. Isolation and Identification of *Pseudomonas aeruginosa*

Water samples were inoculated onto Nutrient Agar and Centrimide Agar. Nutrient agar plates were incubated aerobically at 37°C for 24 hours, while Cetrimide agar plates were incubated at 39°C for 18–48 hours for the selective growth of *P. aeruginosa*. Colonies exhibiting characteristic smooth, flat, blue-green or yellowish-green pigmentation on Centrimide agar were selected for further characterization. Discrete colonies were sub-cultured onto freshly prepared Nutrient and Centrimide agars using sterile wire loops to obtain pure isolates. Colonial morphology was recorded, and isolates were confirmed by Gram staining and standard biochemical tests including oxidase, catalase, and citrate utilization tests, following the criteria of Cheesbrough [10]. Pure isolates were preserved on Nutrient Agar and Centrimide Agar slants at 4°C for subsequent use.

2.4. Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility was determined using the Kirby-Bauer disk diffusion method on Mueller Hinton Agar in accordance with Clinical Laboratory and Standards Institute (CLSI) guidelines. A 0.5 McFarland turbidity standard was used to standardize the inoculum suspension. Bacterial suspensions were swabbed uniformly across the Mueller Hinton agar surface, and antibiotic discs were placed and incubated overnight at 37°C. The following antibiotic discs were tested: Imipenem (10 µg), Amoxicillin/Clavulanic acid (30 µg), Aztreonam (30 µg), Cefoxitin (30 µg), Ceftazidime (30 µg), Oxacillin (10 µg), Gentamicin (30 µg), Amikacin (30 µg), and Ofloxacin (5 µg). Zone of inhibition diameters were measured in millimetres and interpreted as susceptible or resistant per CLSI breakpoints.

2.5. Phenotypic Detection of Beta-Lactamase Enzymes

AmpC β-lactamase Detection: The double disk antagonism test was employed. Ceftazidime (30 µg) and Cefoxitin (30 µg) discs were placed 20 mm apart (centre to centre) on Mueller Hinton agar inoculated with the test isolate. Isolates

showing blunting of the ceftazidime zone of inhibition adjacent to the Cefoxitin disc after overnight incubation were considered positive for AmpC β-lactamase production.

ESBL Detection: ESBL production was confirmed using the combined disk diffusion method per CLSI recommendations. Ceftazidime (30 µg) discs were placed alone and in combination with clavulanic acid. An increase in zone of inhibition of ≥5 mm for Ceftazidime + clavulanic acid compared to Ceftazidime alone was considered indicative of ESBL production.

MBL Detection: Metallo-β-lactamase production was assessed using the Imipenem-EDTA combined disk test. Two 10 µg Imipenem discs were placed on the inoculated plate, and 10 µl of 0.5 M EDTA solution (750 µg) was added to one disc. After incubation at 35°C for 16–18 hours, an increase in zone of inhibition of ≥7 mm for the Imipenem-EDTA disc compared to Imipenem alone was taken as indicative of MBL production. [7]

3. Results

Table 1 Microbial Content of Water Samples Collected from selected river sources in Afikpo LGA., Compared with NSDWQ and WHO Recommended Limits

S/N	Microbial Count (CFU/ml)	Iyi-Oka River	Unwana Beach River	Standard Recommended Limits	
				NSDWQ (2007)	WHO [12]
1.	Total Bacterial X 10^7	3.9 – 12.3	TFTC	100	100
2.	Total Bacterial X 10^6	3.6 – 21.7	TFTC	100	100
3.	Total Bacterial X 10^5	3.4 – 21.1	TFTC	100	100

The total bacterial counts recorded in both Iyi-Oka and Unwana Beach Rivers were considerably higher than the permissible limits of 100 CFU/ml recommended by the NSDWQ [11] and WHO [12], indicating poor microbiological quality. The bacterial counts observed in Iyi-Oka River (3.4×10^5–21.1×10^8 CFU/ml) and the TFTC result obtained for Unwana Beach River suggest heavy microbial contamination, probably due to anthropogenic activities such as indiscriminate waste disposal, agricultural runoff, and faecal pollution. Such elevated bacterial loads may increase the risk of waterborne diseases if the water is consumed without treatment. These findings emphasize the need for regular monitoring and appropriate treatment of these river water sources before domestic use.

Table 2 Morphological and Biochemical Identification of Test Isolates

Colour	Translucency	Shape	Size	Elevation	Consistency	Margin	Oxidase	Catalase	Gram Reaction	Citrate
Blue-green	Opaque	Rod	Moderate	Umbonate	Mucoid	Irregular	+	+	–	+
Yellowish-green	Opaque	Rod	Moderate	Umbonate	Mucoid	Irregular	+	+	–	+
Milky	Opaque	Rod	Moderate	Umbonate	Mucoid	Irregular	+	+	–	+

The table 2 above shows the macroscopic (colonial appearance) characteristics of organism *Pseudomonas aeruginosa* on centrimide selective media. The morphological and biochemical characteristics of the isolates were consistent with those of *Pseudomonas aeruginosa*. The isolates produced blue-green, yellowish-green, or milky opaque colonies with rod-shaped cells, umbonate elevation, mucoid consistency, and irregular margins. They were uniformly oxidase-positive, catalase-positive, Gram-negative, and citrate-positive, confirming the characteristic biochemical profile of *P. aeruginosa.* These findings agree with standard microbiological identification criteria [10] and support the accurate identification of the isolates used in this study.

Table 3 Distribution of *Pseudomonas aeruginosa* Isolated from in selected river sources in Afikpo LGA

Sample Site	No. of Samples Collected from Sites	Number Positive for *P. aeruginosa*	Number Negative for *P. aeruginosa*
Unwana Beach River	10	7 (70%)	3 (30%)
Iyi-Oka River	10	10 (100%)	0 (0%)
Total	20	17 (85%)	3 (15%)

Table 3 shows the percentage distribution of *Pseudomonas aeruginosa* isolated from the rivers sample. Result revealed that of the 20 water samples analyzed, 17 (85%) tested positive for *Pseudomonas aeruginosa*, indicating widespread contamination across both water sources. Iyi-Oka River recorded 100% positivity (10/10 samples), while Unwana Beach River showed 70% positivity (7/10 samples). This overall prevalence is notably higher than rates reported in several comparable studies — for example, 45.4% in a Spanish River study and around 60% for river sources in a Nigerian surface water study — suggesting a heavier bacterial burden at these sites. The complete positivity at Iyi-Oka likely reflects greater human/animal activity, organic load, or environmental conditions favoring bacterial survival, though this would need physicochemical data to confirm. Given that *P. aeruginosa* is an opportunistic pathogen linked to serious infections, its widespread presence in these rivers — especially alongside antimicrobial resistance traits — poses a public health concern for communities relying on these waters, supporting the need for continued surveillance and water safety interventions in Afikpo LGA.

Table 4 Antibiotics Susceptibility Pattern of *Pseudomonas spp*., Isolated from Unwana Beach and Iyi-Oka River in Afikpo North LGA

Antibiotics	Disc Potency (µg)	No. Tested	No. Susceptible (%)	No. Resistant (%)
IMP	10	12	6 (50.00)	6 (50.00)
CAZ	30	12	7 (58.33)	5 (41.67)
AMC	30	12	3 (25.00)	9 (75.00)
OX	10	12	4 (33.33)	8 (66.67)
ATM	30	12	6 (50.00)	6 (50.00)
CN	30	12	12 (100.00)	0 (0.00)
AK	30	12	12 (100.00)	0 (0.00)
FOX	5	12	5 (41.67)	7 (58.33)
OFX	5	12	12 (100.00)	0 (0.00)

Key: IMP = Imipenem, CAZ = Ceftazidime, AMC = Amoxicillin, OX = Oxacillin, ATM = Aztreonam, CN = Gentamicin, AK = Amikacin, FOX = Cefoxitin, OFX=Ofloxacin.

Susceptibility testing of the *Pseudomonas* isolates from Unwana Beach and Iyi-Oka Rivers revealed a mixed resistance profile. High resistance was recorded against amoxicillin/clavulanic acid (75%), oxacillin (66.67%), and cefoxitin (58.33%) — largely consistent with the organism's known intrinsic resistance to these non-anti-pseudomonal agents. More concerning was the 50% resistance to imipenem and aztreonam, and 41.67% resistance to ceftazidime, since these are clinically important anti-pseudomonal drugs; the imipenem resistance rate in particular is notably higher than typically reported even in clinical isolates, hinting at possible MBL-producing strains circulating in these river systems. On a positive note, complete (100%) susceptibility was observed for gentamicin, amikacin, and ofloxacin, meaning these remain fully effective treatment options. Overall, the isolates display a multidrug-resistant (MDR) phenotype, with the carbapenem and monobactam resistance raising public health concern and reinforcing the value of your study's ESBL/AmpC/MBL screening focus.

Table 5 Distribution of AmpC β-lactamase positive, Extended Spectrum Beta-Lactamase and MBL Positive *Pseudomonas aeruginosa* Isolated from Unwana Beach and Iyi-Oka River

Sample Source	AmpC: Number Tested	AmpC: Number Positive (%)	ESBL: Number Tested	ESBL: Number Positive (%)	MBL: Number Tested	MBL: Number Positive (%)
Unwana River	8	5 (62.5%)	6	2 (33.33%)	3	0 (0%)
Iyi-Oka River	10	4 (40.0%)	6	1 (16.67%)	3	0 (0%)

Table 5 shows that AmpC β-lactamase was the predominant resistance mechanism among the *Pseudomonas aeruginosa* isolates, with higher positivity in Unwana Beach River (62.5%) than in Iyi-Oka River (40.0%). ESBL production was less frequent, occurring in 33.33% and 16.67% of isolates from Unwana Beach and Iyi-Oka Rivers, respectively, while no MBL-producing isolates were detected. The predominance of AmpC is consistent with the intrinsic ability of *P. aeruginosa* to produce chromosomally encoded AmpC β-lactamase under selective pressure. [4] Although Iyi-Oka River had a higher overall prevalence of *P. aeruginosa*, Unwana Beach River harboured a greater proportion of resistant isolates, suggesting higher antimicrobial selective pressure. The absence of MBL producers despite phenotypic imipenem resistance observed elsewhere in the study suggests that carbapenem resistance may be mediated by alternative mechanisms such as AmpC overproduction, reduced OprD porin expression, or efflux pump overexpression. [9]

4. Discussion

These findings corroborate previous investigations carried out in Afikpo North [13,17]. The present study demonstrated that water samples from Iyi-Oka and Unwana Beach Rivers were heavily contaminated with bacteria, with total bacterial counts far exceeding the permissible limits recommended by the NSDWQ [11] and WHO [12], indicating poor microbiological quality and potential public health risks. These findings corroborate previous investigations carried out in Afikpo North, which reported elevated heterotrophic bacterial and coliform counts in surface waters used for domestic purposes. Similar observations have also been documented in other communities within Ebonyi State, suggesting that microbial contamination of untreated water sources remains a widespread environmental and public health challenge. [13,17] Morphological and biochemical characterization confirmed the isolates as *Pseudomonas aeruginosa*, and the high prevalence of the organism (85%) in both rivers suggests widespread environmental contamination, possibly resulting from anthropogenic activities such as indiscriminate waste disposal, agricultural runoff, and faecal pollution.[8,9] Antimicrobial susceptibility testing revealed multidrug resistance, with high resistance to amoxicillin/clavulanic acid, oxacillin, cefoxitin, imipenem, aztreonam, and ceftazidime, while gentamicin, amikacin, and ofloxacin remained fully effective against all isolates. [8]

The multidrug resistance pattern observed among the environmental *Pseudomonas aeruginosa* isolates is consistent with previous studies conducted within Afikpo North, where plasmid-mediated resistance was demonstrated among drinking-water isolates of *P. aeruginosa*. Comparable antimicrobial susceptibility profiles have also been reported among clinical isolates recovered from healthcare facilities in Ebonyi State, indicating that environmental water sources may contribute to the persistence and dissemination of resistant strains within the community. [14,16]

AmpC β-lactamase was the predominant resistance mechanism [4], followed by ESBL production [1,3], whereas no MBL-producing isolates were detected. The predominance of AmpC, together with the absence of MBL despite phenotypic imipenem resistance, suggests that carbapenem resistance may be mediated by alternative mechanisms such as AmpC overproduction, reduced OprD porin expression, or efflux pump overexpression. [9] Similar findings have been reported among multidrug-resistant enteric bacteria isolated from drinking-water sources within Afikpo Metropolis, where plasmid-borne resistance determinants were identified among environmental isolates. Collectively, these findings indicate that untreated drinking-water sources in the study area serve as important reservoirs for antimicrobial-resistant bacteria and their associated resistance genes. [15]

5. Conclusion

The findings of this study demonstrate that the selected river sources in Afikpo LGA are microbiologically unsafe for direct domestic use, as they contained bacterial loads far above the limits recommended by the NSDWQ and WHO. A

high prevalence of *Pseudomonas aeruginosa* (85%) was recorded, with many isolates exhibiting multidrug resistance and producing AmpC and ESBL β-lactamases, although no MBL-producing isolates were detected. The predominance of AmpC-mediated resistance suggests that intrinsic resistance mechanisms play a major role in the antimicrobial resistance profile of the isolates. Overall, these rivers constitute potential reservoirs of antibiotic-resistant *P. aeruginosa*, posing a significant public health risk and emphasizing the need for routine microbiological monitoring, improved environmental sanitation, and adequate treatment of river water before human use. [8,9] The present findings complement previous studies conducted within Afikpo North and Ebonyi State, confirming that untreated environmental water sources constitute important reservoirs of multidrug-resistant bacteria and therefore require sustained microbiological surveillance, antimicrobial resistance monitoring, and effective environmental management strategies.

Based on the findings of this study, I recommend that routine microbiological surveillance of Iyi-Oka and Unwana Beach Rivers and other drinking water sources in Afikpo North LGA should be strengthened to monitor bacterial contamination and the emergence of antibiotic-resistant *Pseudomonas aeruginosa*. Untreated river water should be adequately purified before domestic use, while improved environmental sanitation, public health education, and antimicrobial stewardship should be promoted to reduce microbial pollution and limit the spread of antimicrobial resistance. Government agencies should enforce compliance with national and international water quality standards through regular monitoring, and future studies should employ molecular techniques, seasonal sampling, and physicochemical analyses to better understand the distribution, resistance mechanisms, and environmental persistence of *P. aeruginosa* and other waterborne pathogens.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there are no financial or non-financial conflicts of interest in connection with this research. The funding body, TETFund, had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Statement of ethical approval

This study was conducted in accordance with standard microbiological safety guidelines and institutional biosafety protocols of the Microbiology Laboratory Unit, Department of Science Laboratory Technology, Akanu Ibiam Federal Polytechnic, Unwana, Afikpo, Ebonyi State.

Statement of informed consent

As this study involved the collection of environmental water samples from public river sources and did not involve human subjects or animal experimentation, formal ethical approval was not required. However, all laboratory procedures were carried out in strict compliance with established biosafety standards for handling potentially infectious environmental specimens.

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