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Evaluation of Extended Spectrum B-Lactamase producing Enterobacteriaceae from Rivers in Benin City, Nigeria

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Abstract: Extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae is one of the most critical antimicrobial resistant group of bacteria and their presence have been implicated in diverse environmental water and food products. This study aimed at evaluating the prevalence ESBL-producers among Enterobacteriaceae in certain rivers in Benin City, Nigeria. Samples were obtained from three major rivers and evaluated for total heterotrophic bacterial count and Enterobacteriaceae count via culture-based techniques and biochemical protocols. The antibiotic susceptibility profile of the tested isolates was conducted using the disc diffusion protocols of Kirby-Bauer while double disc synergy test was employed in ESBL screening. The total heterotrophic bacteria count of the water samples ranged from 6.67±1.20 - 47.00±1.53 x 10^3 CFU/mL while the total coliform count range from 0.33±0.33 - 14.33±0.33 x 10^3 CFU/mL. The *Escherichia coli* count ranged from 0.33±0.33 - 5.67±0.33 x 10^3 CFU/mL while the *Salmonella* count ranged from 0.31±0.26 - 6.00±1.15 x 10^3 CFU/mL. Other Enterobacteriaceae that was identified include *Citrobacter* spp., *Klebsiella* spp. and *Serratia* spp. The resistance profile of the bacterial isolates showed significant resistance towards ceftazidime, erythromycin, augmentin, cefuroxime and cloxacillin. The prevalence of ESBL-producing Enterobacteriaceae was *Citrobacter* spp. 4/27(14.8%), *E. coli* 3/16(18.8%), *Salmonella* spp. 0/60(0.0%), *Klebsiella* spp. 0/5(0.0%) and Serratia spp. 0/1(0.0%).

1. Introduction

Incessant upsurge in antimicrobial resistance (AMR) remains a foremost biological threat which has adversely contributed to immense decline in the effectiveness of therapeutic options to several bacterial infections in our modern society (Frieri *et al.*, 2017). Bacteria have diverse mechanisms through which they do subdue the efficacy of multiple groups of antimicrobials which includes the enzyme-induced mechanisms. Enzyme-mediated resistance is major public health globally due to its potential to swiftly develop multidrug-resistant (MDR) bacteria according to World Health Organization (WHO) thus causing complications in infection control (Issakhanian & Behzadi, 2019). The extended spectrum β-lactamase (ESBL) strain of Enterobacteriaceae has been declared a major life-threatening group of bacteria among the highest priority pathogens as listed by the WHO (Shrivastava *et al.*, 2018, Murray *et al.*, 2021). Beta-lactamase enzymes are vastly associated with Gram-negative bacteria and they notably promote resistance to most β-lactam drugs including the newer generations.

Globally, ESBL-producing Enterobacteriaceae of healthcare origin has been extensively studied and reported. However, understanding the dynamics of AMR outside the healthcare settings remains a vital tool in the development of sustainable strategies for the surveillance and control of beta-lactam resistance (Destoumieux-Garzón *et al*., 2018). The occurrence of ESBL-producing Enterobacteriaceae even in non-clinical investigations involving natural water bodies and food products has been reported (Subramanya *et al*., 2021). The aquatic environments in particular have been prominently linked to the accretion and dissemination of ESBL-producing Enterobacteriaceae (Cho *et al*., 2023). Surface waters, such as rivers, lakes, and streams are perpetually influenced by anthropogenic activities and these actions have immensely influenced their status as a potential receptacle of AMR determinants. Several water bodies across regions of the world have been implicated as reservoir of ESBL-producing bacteria (Singh *et al*., 2018, Falgenhauer *et al*., 2019) including Nigeria (Obasi *et al*., 2017; Collignon *et al*., 2018).

The persistence of AMR determinants including ESBL-producing Enterobacteriaceae in environmental sources and food products are mostly due to improper sanitation practices and indiscriminate release of poorly treated wastewater into the environment rather than antibiotic abuse (Collignon *et al*., 2018, Koutsoumanis *et al*., 2021). Globally, significant volume of domestically and industrially generated wastewater gets discharged into environmental water untreated or inadequately treated (Montero *et al*., 2021). These wastes are usually associated with AMR genes which could revert back to human system in situations where environmental water contaminated with wastewater are utilized for irrigation and domestic purposes (Pigłowski, 2019). Increased attention has been focused on assessing the presence of AMR genes.

Many antibiotic-resistant enterobacterales can persist and proliferate in the environment and may colonize livestock and human via the fecal-oral route (Basco *et al*., 2015; Guerrero *et al*., 2020). This elevated tendency linked the transfer of AMR genes horizontally within human pathogens intensifies the attention given to the surveillance of AMR-related genes in bacteria of environmental origin (Haberecht *et al*., 2019). All these further emphasized on the significance of piloting routine investigations based on the approach of One Health while considering diverse aspects related to human, animals and environmental health (Destoumieux-Garzón *et al*., 2018). Notably, Enterobacteriaceae remain topmost reported etiological initiator of infections resulting from the ingestion of food products (Motlagh & Yang 2019). The mortality and therapeutic complexity of ESBL-producing Entero-bacteriaceae related infections are predominantly higher when compared to the non-EBSL producing Enterobacteriaceae related infections (Shamsrizi *et al*., 2020). Therefore, this study was aimed at assessing the occurrence of ESBL-producing Enterobacteriaceae in some selected rivers in Benin City, Nigeria.

2. Methodology

2.1 Sample collection

Water samples were obtained from three (3) receiving water bodies namely: Efosa River, Ikpoba Hill River and Okhoro River. These rivers were sampled between the months of July and August 2023 (bi-monthly) using sterile sampling bottles and conveyed on ice packs to Pharmaceutical Microbiology Laboratory, Faculty of Pharmacy, University of Benin, Benin City, for microbiological analysis.

2.2 Enumeration of Enterobacteriaceae in River samples

The water samples were homogenized by agitating the sampling containers after which one (1) mL of each sample was added into nine (9) mL sterile distilled water then diluted in series using the

10-fold dilution protocol. From the diluents, an aliquot of 25µL was spread-plated on nutrient agar for total heterotrophic bacterial count, chromocult coliform agar for total coliform count, eosin methylene blue agar *Escherichia coli* count and deoxychocolate agar for *Salmonella* count. The inoculation was done in triplicates and subsequently incubated for 24 h at 37ºC. The colonies obtained were enumerated and expressed in CFU/mL.

2.3 Characterization and identification of Enterobacteriaceae

Presumptive bacterial colonies were sub-cultured via nutrient agar and determined according to their cultural, morphological and biochemical characteristics. The tests performed were Gram test, motility test, catalase reaction, citrate utilization, coagulase reaction, indole production, methyl-red test, sugar fermentation test and Voges-Proskauer test (Holt *et al*., 1994).

2.4 Determination of antibiotic sensitivity profile of the bacterial isolates

The antibiotic sensitivity profile of isolates was assayed via the Kirby-Bauer disc diffusion techniques. Distinct colonies were suspended in sterile water and matched with Mcfarland standard (0.5). The colony suspensions were aseptically inoculated on sterile Muller Hinton agar plates and evenly distributed on the agar plate using a sterile glass spreader. The antibiotics used include ceftazidime (CAZ, 30 µg), gentamicin (CN, 10 µg), cefuroxime (CRX, 30 µg), cloxacillin (CLA, 10µg), erythromycin (ERY, 15 µg), ceftriaxone (CTR, 30 µg), augmentin (AUG, 30 µg) and ofloxacin (OFL, 10 µg). The antibiotic discs were positioned on the inoculated media using sterile pair of forceps, inverted and incubated for 37ºC for 24 h. The zones as inhibited in diameters were expressed in millimetres with a meter rule. The zones were determined and construed according to the guidelines of clinical laboratory standards institute (CLSI 2020)

2.5 Detection of Extended Spectrum Beta Lactamase-Producing Bacteria Using Double Disc Synergy Test (DDST)

The screening for ESBL was conducted using double disc synergy test as previously described (Weinstein & Lewis 2020). All presumptive isolates were screened for ESBL potentials via the combination disc diffusion protocol using cefotaxime (CFX) (30 µg) and ceftazidime (CAZ) (30 µg) alone and CFX+CAZ in combination with clavulanic acid (30 µg/10 µg) (Difco-BD, Franklin Lakes, NJ, USA). The discs were positioned at 25mm to the centre on Mueller Hilton media seeded with the standardized bacterial suspension and incubated for 18-24 h at 37ºC. The isolates were classified as ESBL-producers when an inhibition zone greater than 5mm is observed in media culture with or without clavulanic acid. The control strains used were ESBL-positive *Klebsiella pneumoniae* ATCC 700603 and ESBL-negative *Escherichia coli* ATCC 25922.

2.6 Data Analysis

The mean and standard error of triplicate results of the bacterial counts of the isolates were analyzed using SPSS. Analysis of Variance (ANOVA) was also used to determine significant difference in results when necessary.

3. Results and Discussion

3.1 Enumeration of Bacteria in Selected Rivers

The enumeration of bacteria in the rivers revealed that there was varying microbial count of bacteria, coliform, *E. coli* and *Salmonella* count throughout the period of study. Table 1 shows the total

heterotrophic bacteria count of the river and the highest bacteria counts (47.00±1.53 x 10^3 CFU/mL) was recorded in Okhoro water and Efosa water samples in week 6. The least value (6.67±1.20 x 10^3 CFU/mL) was recorded in week 0 from Efosa water sample. Table 2 revealed the total coliform count of the river and the highest coliform counts were recorded at week 6 in Efosa water sample (14.33±0.33 x 10^3 CFU/mL) while the least value was recorded in week 0 from Efosa water (0.33±0.33 x 10^3 CFU/mL).

Table 1. Total heterotrophic bacterial count of the river

Period of Study (Weeks)	Bacterial load (x 10^3 CFU/mL)		
	Efosa	Okhoro	Ikpoba
0	6.67±1.20^a	47.00±1.53^c	29.33±2.60^b
2	9.00±1.53^a	23.33±1.20^b	24.00±1.73^b
4	16.67±1.20^a	17.33±1.20^a	32.00±0.57^b
6	47.00±1.53^c	25.33±2.33^b	15.67±0.33^a
8	39.67±1.45^c	24.00±2.30^b	13.67±1.45^a

*Values are the mean and standard error of triplicate; a-c: different characters in the same column indicate values with significant difference (p>0.05).

Table 2. Total coliform count of the river

Period of Study (Weeks)	Coliform Count (x 10^3 CFU/mL)		
	Efosa	Okhoro	Ikpoba
0	0.33±0.33^a	12.67±0.67^c	5.67±0.88^b
2	2.33±0.33^a	3.67±0.33^a	5.33±0.88^b
4	8.33±0.88^b	3.00±1.00^a	8.33±0.88^b
6	14.33±0.33^b	14.67±0.67^b	8.33±0.33^a
8	14.00±0.00^b	16.67±1.20^c	2.00±0.00^a

*Values are the mean and standard error of triplicate; a-c: different characters in the same column indicate values with significant difference (p>0.05).

Figure 1 shows the *E. coli* count of the rivers where the highest *E. coli* counts was observed at week 4 in Ikpoba water sample (5.67±0.33 x 10^3 CFU/mL) while the least value was in week 0 from Efosa water (0.33±0.33 x 10^3 CFU/mL). Figure 2 shows the *Salmonella* count of the rivers and the highest *Salmonella* count was recorded at week 0 in Ikpoba water sample (6.00±1.15 x 10^3 CFU/mL) while the least value was also recorded in week 0 water sample collected from Efosa water (0.31±0.26 x 10^3 CFU/mL).

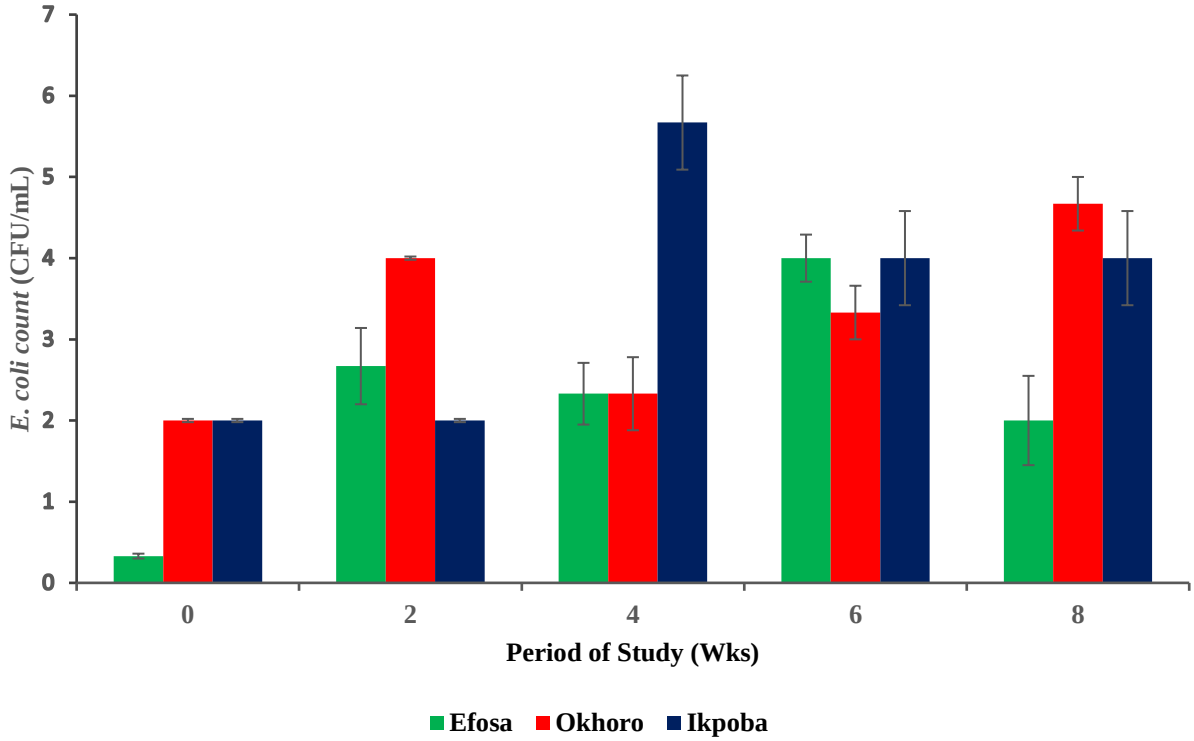

Figure 1. *Escherichia coli* count of the river**.**

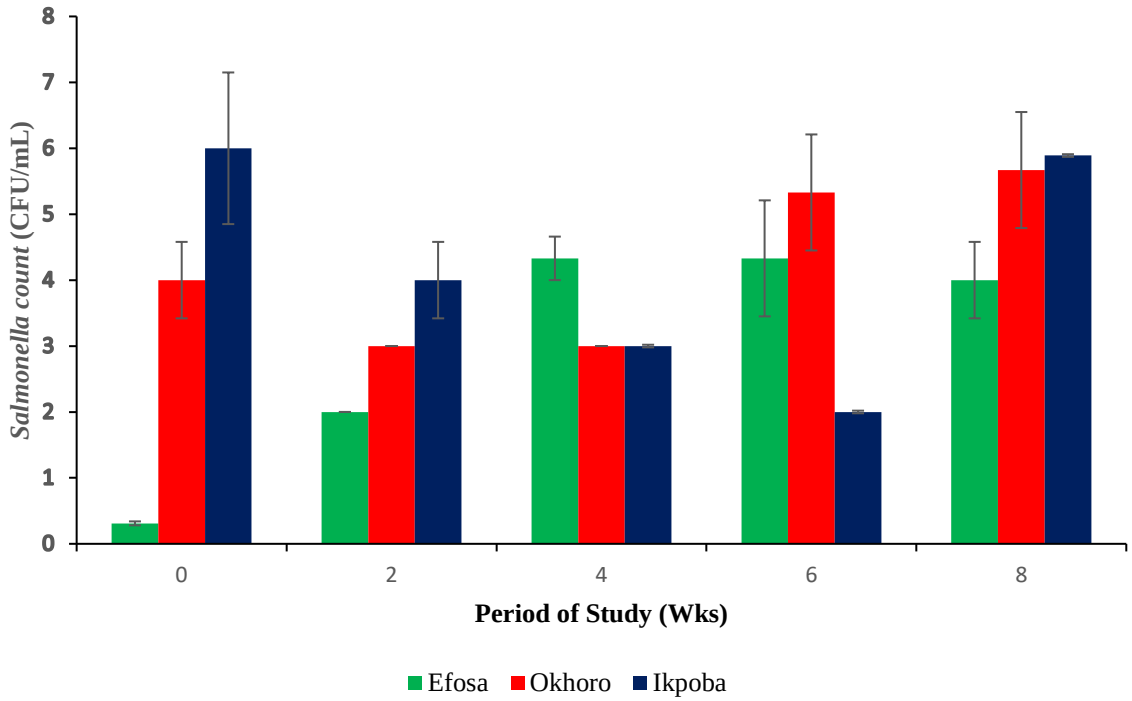

Figure 2: *Salmonella* count in the rivers

In human nutrition, water remains very essential either as constituent of food or via direct consumption. However, vast proportion of individuals residing in low-income countries remains primarily dependent on surface water (Adesakin *et al*., 2020). In this study, the total heterotrophic bacteria counts (THBC) of the water samples range from 6.67±1.20 - 47.00±1.53 x 10^3 CFU/mL while the total coliform counts range from 0.33±0.33 - 14.33±0.33 x 10^3 CFU/mL. This is higher than THBC

mean values that range from 1.7×10^3 - 2.7×10^3 CFU/mL in water samples from lake, borehole and stream (Akrong *et al*., 2019). However, this is lower that than the THBC recorded in previous study on domestic water sources which range from 8.49×10^5 - 6.02×10^7 (Adesakin *et al*., 2020). Nevertheless, they all exceeded the permissible limits based on WHO recommendations of <500 CFU/mL in drinking water sources (WHO 2011). Elevated bacteria population in the open water sources could have resulted from their exposed surfaces which increased their contact tendencies with pollutants from runoffs and anthropogenic actions such as laundries, swimming and domestic effluents (Amu-Mensah *et al*., 2014). The coliform count observed in this study is comparable to the mean coliform counts recorded in stream water from previous studies as both exceeded the WHO recommended limit for water meant for domestic usage (Akrong *et al*., 2019). Coliforms are primary indicator for water pollution by faecal matters and they are one of the prominent bacteria in associated with waterborne diseases globally (WHO 2011). Based on WHO recommendations, the permissible limits of coliform should be zero per 100mL in drinking water while the limit in water used for recreational services should be 126 CFU/100 mL. (Gunda &and Mitra 2016).

3.2 Identification and Antibiotic susceptibility evaluation of Enterobacteriaceae isolates

Table 3 shows the Enterobacteriaceae isolates identified from the selected rivers. These include *Escherichia coli*, *Citrobacter* spp., *Klebsiella* spp., *Salmonella* spp., and *Serratia* spp. The distribution of occurrence from the rivers was Efosa River (*Citrobacter* spp. and *Escherichia coli*), Okhoro River (*Citrobacter* spp., *Salmonella* spp. and *Escherichia coli*) and Ikpoba Hill River (*Citrobacter* spp., *Klebsiella* spp., *Escherichia coli*, *Salmonella* spp. and *Serratia* spp.).

The resistance profile of the bacterial isolates in percentage (%) was shown in Table 3. The *Citrobacter* isolates from Efosa River demonstrated absolute resistance (100%) towards augmentin while 83.3% of the *Citrobacter* isolates from Efosa River were resistant towards ceftazidime and erythromycin. The least resistance was towards ofloxacin and gentamicin. The *E. coli* isolated from Efosa River revealed absolute resistance (100%) against augmentin, cefuroxime, cloxacillin and erythromycin (Taibi *et al*., 2024). None of the tested *E. coli* isolates from Efosa River demonstrated resistance towards ofloxacin and gentamicin.

The *Citrobacter* and *E. coli* isolates from Okhoro River demonstrated absolute resistance (100%) towards ceftazidime, ceftriaxone, erythromycin, cloxacillin and augmentin. However, the isolates demonstrated no resistance towards ofloxacin. The tested *Salmonella* isolates from Okhoro River was 100% resistant to cloxacillin and augmentin while none was resistant towards other tested antibiotics with the exception of erythromycin which had 50% resistant rate. The resistance profile of bacterial isolates from Ikpoba Hill River showed that all the tested isolates demonstrated 100% resistance to erythromycin, cloxacillin and augmentin while none of the isolates recorded resistance against ofloxacin.

This study demonstrated the detection of Enterobacteriaceae including *Escherichia coli*, *Klebsiella* spp., *Citrobacter* spp., *Salmonella* spp. and *Serratia* spp. These genera linked to the investigated water could have resulted from agricultural activities around water bodies and other anthropogenic activities. Previous studies have attributed microbial infiltration in surface water, a phenomenon that significantly influence the spread of infectious diseases to human activities such as washing, bathing, farming and seepages of animal/human feces into water bodies via runoffs (Anyanwu and Okoli 2012; Alegbeleye & Sant'Ana, 2020; Alaqarbekh *et al.*, 2021; Akhtar *et al.*, 2021; Saidi *et al.*, 2022).

Table 3. Enterobacteriaceae isolated from the selected river and their resistance profile

Location (River)	Bacterial Isolates	Resistance Profile (%)								
		N	CAZ	CRX	GEN	CTR	ERY	CXC	OFL	AUG
Efosa	***Citrobacter* spp.**	6	83.33	33.33	16.67	66.67	83.33	83.33	16.67	100.0
	Escherichia coli	4	75.00	100.0	0.00	75.00	100.0	100.0	0.00	100.0
Okhoro	***Citrobacter* spp**	5	100.0	60.00	20.00	100.0	100.0	100.0	0.00	100.0
	Escherichia coli	9	100.0	22.22	33.33	100.0	100.0	100.0	11.11	100.0
	***Salmonella* spp.**	2	0.00	0.00	0.00	0.00	50.00	100.0	0.00	100.0
Ikpoba Hill	***Citrobacter* spp**	16	100.0	25.0	37.50	62.50	100.0	100.0	0.00	100.0
	Escherichia coli	3	33.33	33.33	33.33	33.33	100.0	100.0	0.00	100.0
.	***Salmonella* spp**	4	100.0	25.00	50.00	100.0	100.0	100.0	0.00	100.0
.	***Klebsiella* spp**	5	80.00	40.00	(20.00	80.00	100.0	100.0	0.00	100.0
	***Serratia* spp.**	1	100.0	100.0	0.00	100.0	100.0	100.0	0.00	100.0

Key: N: Number of isolates tested; CRX: Cefuroxime; CAZ: Ceftazidime; CN: Gentamicin; ERY: Erythromycin; CTR: Ceftriaxone; OFL: Ofloxacin; CXC: Cloxacillin; AUG: Augmentin

The resistance profile of bacterial isolates was significantly resistant to augmentin, ceftazidime, erythromycin and cloxacillin. The *Citrobacter* isolates from Efosa River were all resistant towards augmentin while ofloxacin and gentamicin were the most effective antibiotics. The *E. coli* isolates from Efosa River were also all resistant towards augmentin, cefuroxime, cloxacillin and erythromycin with no resistance towards ofloxacin and gentamicin. Similarly, *Citrobacter* and *E. coli* isolates from Okhoro River were absolutely resistant towards ceftazidime, ceftriaxone, erythromycin, cloxacillin and augmentin with no resistance towards ofloxacin. Resistance profile of all bacterial isolates from Ikpoba Hill River also demonstrated total resistance to erythromycin, cloxacillin and augmentin, although none of the isolates recorded resistance against ofloxacin. These findings agree with previous studies that reported significant antibiotic resistance in Enterobacteriaceae isolates from domestic and irrigation water sources (Vital *et al*., 2018, Ortega-Parede *et al*., 2020). High prevalence of drug-resistant bacteria in vegetables farms has also been emphasized (Araújo *et al*., 2017, Diab *et al*., 2018).

3.3 Determination of ESBL-producing Enterobacteriaceae via Double Disc Synergy Test (DDST)

Table 4 shows the prevalence of ESBL-producing Enterobacteriaceae as evaluated using DDST. The prevalence as observed was *Citrobacter* spp. 4/27(14.8%), *E. coli* 3/16(18.8%), *Salmonella* spp. 0/6(0.0%), *Klebsiella* spp. 0/5(0.0%) and *Serratia* spp. 0/1(0.0%).

Table 4. Detection of ESBL-Producing Bacteria in selected rivers

Bacteria Isolates	Number Tested	EBSL-Positive n (%)
Citrobacter spp.	27	4 (14.8)
Escherichia coli	16	3 (18.8)
Salmonella spp.	6	0 (0.0)
Klebsiella spp.	5	0 (0.0)
Serratia spp.	1	0 (0.0)

3.4 Antibiotic resistance profile and MAR index of EBSL-Positive isolates

The antibiotic resistance profile and MAR index of EBSL-positive isolates was revealed in Table 5. The data showed that the MAR index ranged from 0.25 to 0.75. The findings of this study showed that *Citrobacter* spp. isolated from Ikpoba Hill water had the highest MAR index of 0.75 with resistance against 6 antibiotics (ceftazidime, cefuroxime, ceftriaxone, erythromycin, cloxacillin and augmentin). The least MAR index was also recorded in *Citrobacter* spp. isolate from Okhoro River with resistance against 2 antibiotics (erythromycin and cloxacillin). All the tested isolates were resistant to erythromycin and cloxacillin while they were all susceptible to gentamicin. In this study, ESBL-production was detected in *E. coli* 3/16(18.8%) and *Citrobacter* spp. 4/27(14.8%) while ESBL-production was undetected in all *Salmonella* spp., *Klebsiella* spp. and *Serratia* spp. isolates tested. This agrees with previous study that reported *E. coli* to be the most common host among the wide range of Gram-negative bacterial species which harbors the ESBL genes (Diab *et al*., 2018). The detection of ESBL-producing *Klebsiella* was however not observed in this study as contrarily observed in other reports (Diab *et al*., 2018; Husna *et al*., 2023). The activities of ESBL-producing Enterobacteriaceae in aquatic bodies have been detected in Europe (Zarfel *et al*., 2017), Asia (Gomi *et al*., 2017), South America (Bastidas-Caldes *et al*., 2022) and several other regions of the world.

Table 5. Antibiotic resistance profile and MAR index of EBSL-Positive isolates

Isolate source	Organism	Antibiotics Susceptibility Profile								MAR Index
		CAZ	CRX	CN	CTR	ERY	CXC	OFL	AUG	
Efosa	*Escherichia coli*	S	S	S	S	R	R	R	R	0.50
Okhoro	*Escherichia coli*	S	R	S	S	R	R	S	R	0.50
Okhoro	*Escherichia coli*	S	S	S	S	R	R	S	R	0.38
Okhoro	*Citrobacter* spp.	R	S	S	S	R	R	S	S	0.38
Okhoro	*Citrobacter* spp.	S	S	S	S	R	R	S	S	0.25
Ikpoba	*Citrobacter* spp.	R	R	S	R	R	R	S	R	0.75
Ikpoba	*Citrobacter* spp.	S	R	S	S	R	R	S	R	0.50

Key: S: Sensitive; R: Resistant; CRX: Cefuroxime; CTR: Ceftriaxone; CAZ: Ceftazidime; CN: Gentamicin; ERY: Erythromycin; AUG: Augmentin; OFL: Ofloxacin; CXC: Cloxacillin.

In Africa, the presence of surface-water related ESBL-producing Enterobacteriaceae has been detected in Tunisia (Hassen *et al*., 2018), Tanzania (Baniga *et al*., 2020), South Africa (Nzima *et al*., 2020), Congo DR (Laffite *et al*., 2020), Ghana (Banu *et al*., 2021) and Nigeria (Atta *et al*., 2021). Furthermore, the occurrence of ESBL-producers has been reported in aquatic animals (Sapugahawatte *et al*., 2020, Jinnai *et al*., 2024) and also in vegetables meant for human consumption (Montero *et al*., 2021). The presence of ESBL-producing bacteria in food producing animals and food crops further intensifies the risk of food to human transfer of these resistance traits (Murray *et al*., 2021). This situation signals potential risks associated with the dissemination of bacteria strains that could be resistant to last-line antibiotics (Sheu *et al*., 2019). These findings aggravate the risks of ESBL-producing bacteria spread through domestic water and other food sources.

Conclusion

ESBL-producing bacteria have become a widespread concern and its presence in the environment. The health challenges associated with the widespread dissemination of ESBL-producers are enormous and these include complications in the management of related infections as it limits the effectiveness of several antibiotics. In view of these challenges, it is imperative to curtail the distribution of ESBL-related bacteria and other determinants of antibiotic resistance. These measures include the improved sanitary practices prudent use of antibiotics in agriculture. In conclusion, the high bacteria counts observed in the river sources investigated notably make them bacteriologically unsafe for drinking purposes.as they exceeded the safe limits as recommended.

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