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Multiple Antimicrobial Resistance in *Vibrio* spp Isolated from River and Aquaculture Water Sources in Imo State, Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author CIC designed the protocol, carried out the laboratory analyses and wrote the first draft of the manuscript. Authors SNI and GCO supervised the work, analysed the results and edited the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: To study multiple antimicrobial resistances in *Vibrio* spp. isolated from river and aquaculture water sources in Imo State Nigeria.
Methodology: A total of 157 *Vibrio* isolates from river and aquaculture water sources were analysed for multiple antimicrobial resistance during a 6 month period. Antimicrobial resistance profile was determined by the Kirby-Bauer technique, while the phenotypic expression of β-lactamase production was performed by the double disk diffusion method. PCR was used to screen isolates for the presence of β-lactamase resistance genes.
Results: The isolates from river water expressed high resistance rates (81.3 to 97.8%) to the following antimicrobials: mezlocillin, doxycycline, tetracycline, carbenicillin and ampicillin, while resistance rate to kanamycin was moderate at 40.9%. Resistance rates for the aquaculture water Isolates were also high for the same antibiotics as the river water isolates, while resistance rate to kanamycin was low to moderate at 32.8%. Phenotypic screening of isolates for ESβL production showed the isolates were resistant to β-lactam antimicrobials and the β-lactamase inhibitor of amoxicillin/clavulnic acid combination. Gel electrophoresis of PCR products showed amplification for bla_{TEM} of size 964bp.

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Conclusion: Results showed the presence of highly resistant *Vibrio* isolates from the sampled environmental sources. The presence of resistance markers among the isolates in this study infers that they could be agents of transfer of resistance to other bacterial pathogens found in river and aquaculture water.

Keywords: Vibrio sp; bla$_{TEM}$; β-lactamase; antimicrobial resistance.

1. INTRODUCTION

Bacterial pathogens, once susceptible to antimicrobial agents are becoming increasingly resistant. In addition, hospitals are now facing problems caused by new opportunistic pathogens (from non-clinical environments) that show little susceptibility to antibiotics [1,2]. Increasing antibiotic resistance poses important risks to human health [3] and can affect the course of infectious diseases [4], increasing the danger associated with immuno suppression (e.g. in transplantation and anti-cancer chemotherapy), intubation, catheterization and other common procedures, all of which rely on antibiotics to overcome the infections with which they are commonly associated.

The emergence of bacterial resistance to antibiotics is common in areas where antibiotics are used, but antibiotic resistant bacteria also increasingly occur in aquatic environments [5,6]. The widespread use of antibiotics in medicine and intensive animal husbandry is indicative of the selective pressure exerted on bacteria [7]. Antibiotic resistant bacteria [7, 8] and antibiotics [9] are discharged in various amounts in the environment as a result of the increasing and often indiscriminate use of antibiotics in medical, veterinary and agricultural practices.

River waters are the main receptacles for these pollutants, since they receive the sewage of urban effluents. As river bodies are one of the major sources of water directly or indirectly for human consumption, this pollution may contribute to the maintenance and even the spread of bacterial antibiotic resistance [10]. The presence of antibiotics in the aquatic environment can result in the appearance of resistance among human pathogens forming part of its micro biota [11]. Consequently, bacteria resistant to antibiotics have been found in surface water [12,13]. In addition, bacteria with intrinsic or acquired resistance to antibiotics have commonly been found in aquatic environments, where *Pseudomonas, Serratia*and *Aeromonas* are commonly identified [14,15].

Vibrios are natural inhabitants of aquatic environments with high salinity and temperatures ranging from 10 to 30ºC [16,17]. Several species, including *V. harveyi, V. parahaemolyticus, V. alginolyticus, V. anguillarum* and *V. splendidus*, are known to induce severe infections in aquaculture livestock, especially shrimp [18]. Several vibrios are also important human pathogens. *Vibrio cholerae*causes cholera and *V. parahaemolyticus* can cause gastroenteritis in humans following consumption of contaminated sea food [19]. Other vibrios that have been implicated in diseases of humans include: *V. mimicus, V. vulnificus, V. hollisae*and *V. fluvialis* that also cause acute gastroenteritis when contaminated sea foods are consumed. Others include *V. alginolyticus* and *V. damsela.* These cause eye sore or wound infections after exposure to sea water [20].

Chemicals and antibiotics are widely used to prevent or treat such infections. However, according to Nogueira-Lima et al. [21] evaluating the risks associated with the use of chemicals in aquaculture is difficult due to the lack of quantitative data from most countries

involved in this activity. Most available information on metabolization efficiency, tissue withdrawal time and environmental impact has been collected in temperate climate zones and may not apply to tropical environments and species [22].

Most research work carried out with Nigeria as case study has been on *V. cholera* isolates from clinical samples [23] and review on cholera epidemiology [24]. This study is the first account to the best of our knowledge, of analysis of antimicrobial resistance in *Vibrio* spp isolated from environmental sources in Nigeria. This study was therefore undertaken to study multiple antimicrobial resistance in *Vibrio* spp isolated from river and aquaculture water sources and also determine possible role of β-lactamase genes in identified resistance traits.

2. MATERIALS AND METHODS

2.1 Study Areas

River water samples were collected from one of two rivers, Nworie River, that border Owerri metropolis in Imo State Nigeria. The river runs about a 5km course across the town. The river is exposed to intensive human and industrial activities resulting in the discharge of a wide range of pollutants, including sewage. Nworie River receives effluents from the Federal Medical Centre and AlvanIkoku Federal College of Education Owerri, both of which lie along its course. The river also serves as a drinking water source for inhabitants when the public water supply fails. Water samples were collected from four (4) sites along the River course, from the upstream to downstream points. The aquaculture water samples were collected from private and commercial fish ponds located elsewhere in Owerri metropolis.

2.2 Sample Types, Processing and Bacterial Isolates

Water samples for analysis were collected from river water and aquaculture sources in sterile 1L sample containers. Initial bacterial isolation was done by inoculating 1ml of the samples into 5mls of sterile alkaline peptone water (Oxoid) for enrichment of *Vibrio* sp. and tubes were incubated for 24h at $28^{o}\pm2^{o}$C. After incubation, 0.1ml of enriched samples were spread on the dry surfaces of Thio Citrate Bile Salts Sucrose (TCBS) (Lab M) agar plates for the isolation of both sucrose and non-sucrose fermenting *Vibrio* isolates. Isolates were confirmed with API 20 NE (Bio-Mérieux,).

2.3 Antimicrobial Resistant Testing and Screening for EsβIs

Routine antibiograms were determined by the Kirby-Bauer disk diffusion technique on Mueller-Hinton agar (Oxoid, Basingstoke). The antibiotics assayed and their concentrations were ampicillin (10µg), mezlocillin (75µg) kanamycin (30µg), tetracycline (30µg), carbenicillin (100µg) and doxycycline (30µg) (Oxoid, Basingstoke). The results were interpreted according to the criteria established by the CLSI [25]. Phenotypic expression of ESBL production was conducted by the double disk diffusion test with the following antimicrobials: imipenem (10 µg), cefpodoxime (10 µg), cefepime (30 µg), cefpirome (30 µg), amoxicillin +clavulanic acid (20/10µg) (MAST Diagnostics, England).

2.4 Test for Beta-lactamase Production

Beta-lactamase production was confirmed by the Nitrocefin test [26]. The Nitrocefin test in this study was performed using Nitrocefin discs (Mast discs™ ID) and was used as a confirmatory test for the production of beta lactamase by the *Vibrio* isolates.

2.5 PCR Detection of β-lactamase Genes

Bacterial chromosomal DNA was used as substrate for the PCR and specific primers for the β-lactamase genes TEM, SHV and CTX [27,28,29], were used for the detection of resistance genes (Table 1.). PCR was performed in 25 µl of a reaction mixture containing DNA (10-200ng), 200 µM of each deoxynucleoside triphosphates (dNTP) (Promega), 1.5 mM $MgCl_2$, 1X PCR Buffer, 20 pMol (each) of the primers, 1 unit of *Taq DNA* polymerase (Promega) and sterile distilled water. Thermal cycling was conducted in an Eppendorf Master Cycler Gradient at an initial denaturation temperature of 94ºC for 5 minutes, followed by 30 amplification cycles of 1 minute at 94ºC; 1 minute at (50ºC for bla_{TEM}, 54ºC for bla_{SHV} and 62ºC for bla_{CTX}) and 1 minute at 72ºC. This was followed by a final extension step of 10 minutes at 72ºC. The amplification product was separated on 1.5% agarose gel and visualized by staining with ethidium bromide. One hundred (100) bp DNA ladder was used as DNA molecular weight standard.

Table 1. The primers used to screen for ESβL genes

Primer	Gene	Sequence/size (bp)	Reference
757(FP)	bla_{TEM}	5'-GCGGAACCCCTATTTG-3' / 964	27
821(RP)	bla_{TEM}	5'-TCTAAAGTATATATGAGTAAACTTGGTCTGAC-3'/964	
1436(FP)	bla_{SHV}	5'-TTCGCCTGTGTATTATCTCCCTG-3' / 854	28
1437(RP)	bla_{SHV}	5'-TTAGCGTTGCCAGTGYTCG-3' / 854	
1354(FP)	bla_{CTX}	5'-ATGTGCAGYACCAGTAARGTKATGGC-3' / 593	29
1355(RP)	bla_{CTX}	5'-TGGGTRAARTARGTSACCAGAAYCAGCGG-3' / 593	

2.6 Statistical Analysis

Differences in results were analysed for significance using SPSS 16.0 for Windows.

3. RESULTS AND DISCUSSION

A total of 157 isolates of *Vibrio* sp. were isolated from 50 river and aquaculture water samples. Three genera of vibrios as confirmed with API 20 NE were identified among the environmental isolates and these included *Vibrio cholerae, V. parahaemolyticus*and *V. vulnificus*.

Antimicrobial resistance screening showed that the river water isolates expressed high resistance rates ranging from 81.3% to 97.8% for all the antimicrobials tested except for Kanamycin that recorded 40.9% (Table 2.). A comparative analysis of resistance rates between the vibrios isolated from river water showed that the *Vibrio* sp. expressed higher resistance rates than the *V. cholerae* in 4 of the 6 antimicrobials tested. Resistance rates to carbenicillin and kanamycin were higher in *V. cholerae* (Fig. 1.).

Table 2. Frequency of antimicrobial resistance of *Vibrio* sp. isolated from river water

Antibiotics	No (%) of resistant isolates				Total no. of isolates	Total % resistance
	No. of isolates	*Vibrio cholerae*	No. of isolates	*Vibrio* sp		
AMP	77	75(97.4)	16	16(100)	93	91(97.8)
CAR	77	70(90.9)	16	14(87.5)	93	84(90.3)
TE	77	68(88.3)	16	16(100)	93	84(90.3)
DO	77	61(79.2)	16	15(93.8)	93	76(81.7)
MEZ	32	25(78.1)	16	14(87.5)	48	39(81.3)
K	77	32(41.6)	16	6(37.5)	93	38(40.9)

KEY: AMP: Ampicillin; CAR;carbenicillin; TE: tetracycline; DO: doxycycline; MEZ:mezlocillin; K: kanamycin

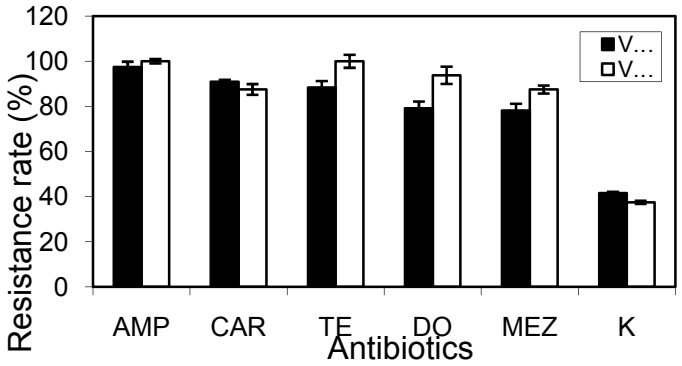

Fig. 1.Comparative analysis of rates of resistance between *Vibrio* sp. from river water samples. Error bars represent the standard error of the mean

KEY: V.ch: Vibrio cholerae; V. sp: Vibrio sp.; AMP: Ampicillin; CAR:carbenicillin; TE: tetracycline; DO: doxycycline; MEZ:mezlocillin; K: kanamycin

For the aquaculture isolates, a similar trend was also noticed, with high resistance rates ranging between 70 and 98.4% for five of the tested antimicrobials, while resistance rates to kanamycin was low to moderate at 32.8% (Table 3.). Also a comparative analysis of resistance among the aquaculture isolates showed higher resistance rates among the *V. cholerae* to 4 of 6 antimicrobials tested. Resistance rate to kanamycin was higher with the *V. cholerae* isolates than the *Vibrio* spp (Fig. 2).

Table 3. Frequency of antimicrobial resistance of *Vibrio* sp. isolated from aquaculture water

Antibiotics	No (%) of resistant isolates				Total no. of isolates	Total % resistance
	No. of isolates	*Vibrio* sp.	No. of isolates	*Vibrio cholerae*		
AMP	47	46(97.9)	17	17(100)	64	63(98.4)
TE	47	40(85.1)	17	16(94.1)	64	56(87.5)
DO	47	34(72.3)	17	17(100)	64	51(79.7)
CAR	47	36(76.6)	17	13(76.5)	64	49(76.6)
MEZ	12	7(58.3)	8	7(87.5)	20	14(70.0)
K	47	16(34.0)	17	5(29.4)	64	21(32.8)

KEY: AMP: Ampicillin; CAR:carbenicillin; TE: tetracycline; DO: doxycycline; MEZ: mezlocillin; K: kanamycin.

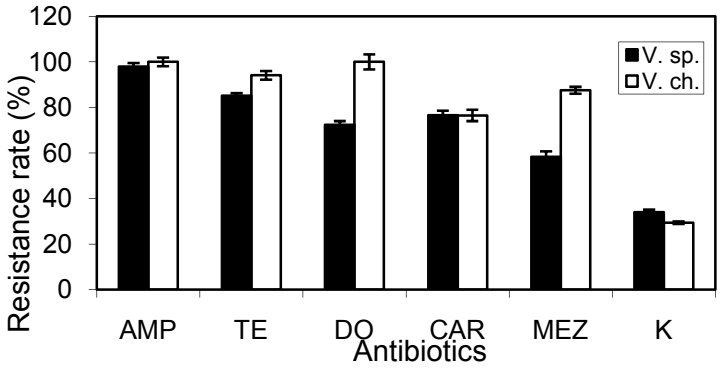

Fig 2. Comparative analysis of rates of resistance between *Vibrio* sp. from aquaculture water samples. Error bars represent the standard error of the mean

KEY: V. ch.: Vibrio cholerae; V. sp.: Vibrio sp.; AMP: Ampicillin; CAR:carbenicillin; TE: tetracycline; DO: doxycycline; MEZ:mezlocillin; K: kanamycin

Generally however, the river water isolates expressed higher resistance rates than the aquaculture isolates to 5 of the antimicrobials, while the aquaculture isolates expressed a slightly higher resistance rate for ampicillin (Fig. 3.). These differences were found significant (*P* =.05) at 95% confidence interval.

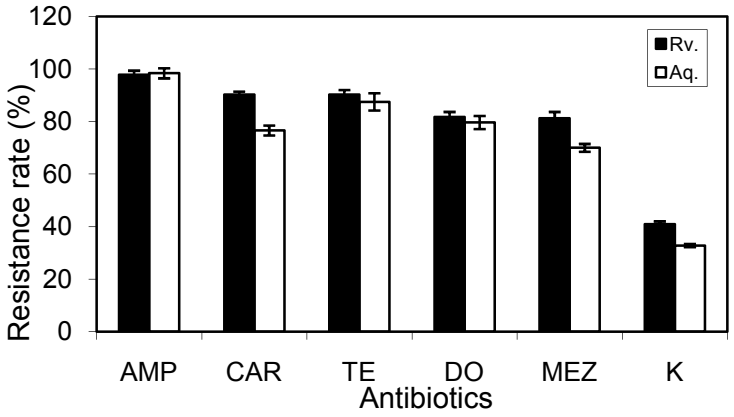

Fig. 3. Comparative analysis of rates of resistance between *Vibrio* sp. from the river and aquaculture water samples. Error bars represent the standard error of the mean

KEY: Rv: river water; Aq: aquaculture water; AMP: Ampicillin; CAR:carbenicillin; TE: tetracycline; DO: doxycycline; MEZ:mezlocillin; K: kanamycin

Aeromonas sp., non-cholera vibrios and *Plesiomonasshigelloides* belong to the expanding group of water and food borne pathogens. They are widely distributed in aquatic environments and are increasingly regarded as important pathogens of aquatic animals, causing significant economic losses in the aquaculture industry worldwide. In addition, these bacteria have been implicated as opportunistic pathogens mainly causing gastroenteritis in humans [30]. Antibiotic resistant *Vibrio cholerae* strains are increasingly being found worldwide [31,32,33,34,35].

Analysis of the antimicrobial resistance tests of the *Vibrio* spp shows that the isolates presented high rates of resistance to ampicillin (97.8% and 98.4%) for both river and

aquaculture water samples respectively. Resistance rates to carbenicillin, tetracycline, doxycycline and mezlocillin were also high at between 81% and 90.3% for both river and aquaculture water samples respectively. Resistance rates to kanamycin was however moderate for isolates from both environmental samples.

In similar studies, Akinbowale *et al.* [36] also found widespread resistance to ampicillin in his study of aquaculture isolates and Campos *et al.* [33] reported 64% resistance to ampicillin in their environmental non-O1 *Vibrio* strains. The rates of resistance in the environmental *V. cholerae* isolates in the present work correlates with resistance rates of clinical *V. cholerae* O1 strains (83%) of Campos *et al.* [33] for ampicillin. According to him one can assume that some of the environmental O1 isolates with antibiotic profiles similar to clinical strains represent strains that have been shed into the environment from clinical cases. The river receives effluents and sometimes raw sewage from the health and academic institutions that lie along its course. These wastes could be sources of resistant clinical isolates into the water body, which can in turn transfer resistant characters to other bacterial species.

Total resistance rates to tetracycline at 90.3% and 87.5% for river and aquaculture water isolates respectively is worrisome and of great concern because tetracycline is the antibiotic of choice for the treatment of cholera [37]. Tetracycline- resistant *V. cholerae* O1 strains have been responsible for major epidemics around the world [31,38].

All the isolates from both environmental sources were resistant to at least two or more antimicrobials with about 30 isolates being resistant to all 5 antimicrobials. High levels of multiple resistances were exhibited by the *Vibrio* isolates in this study, which agrees with the results of the studies conducted by Akinbowale *et al.* [36] and Amaro *et al.*[39]. Multiple drug resistance has also been reported in a number of studies of fish pathogens and aquaculture environments [40,41,42]. *Vibrio* spp isolated from shrimp hatcheries in Indonesia has demonstrated multiple antimicrobial resistance to antimicrobials such as ampicillin, tetracycline, amoxicillin and streptomycin [22,43,44] and Livermore[45] reported that bacteria resistant to six to ten antimicrobials were common.

One *Vibrio* isolate from the aquaculture samples showed amplification of the bla_{TEM} lactamase gene at 964bp. Plasmid mediated bla_{TEM} and bla_{SHV} are the most common genes encoding beta-lactamases and extended-spectrum beta-lactamases, a major cause of resistance to beta-lactams, and they are increasingly being found in different settings worldwide [33,46]. The enzymes encoded by these genes confer unequivocal resistance to ampicillin, amoxicillin, ticarcillin and carbenicillin [47,48].

4. CONCLUSION

The study showed the presence of multiple resistant *Vibrio* isolates as well as genes encoding ESβLs in isolated species in the environmental samples studied. Non-pathogenic *Vibrio* species have been found to be useful and applied as probiotics in aquaculture, and thus, may pose a risk to the fish industry, with respect to the transfer and maintenance of resistance traits in the fish and water environments.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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