

Research Paper

Multiple Antimicrobial Resistance in *Vibrio* sp isolated from River and Aquaculture Water Sources in Imo State, Nigeria.

ABSTRACT

Aim: To study multiple antimicrobial resistance in *Vibrio* sp. isolated from river and aquaculture water sources in Imo State Nigeria.

Methodology: A total of 157 *Vibrio* isolates 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 -lactam resistance markers.

Results: Three genera of vibrios including *Vibrio cholera*, *V. parahemolyticus* and *V. fulniscus* were isolated as confirmed by API 20 NE. The isolates from river water expressed high resistance rates (81.3 – 97.8%) to 83% of tested antimicrobials. Isolates from the aquaculture water also expressed high and moderate resistance rates to 83.3% and 16.7% of the tested antimicrobials respectively. Phenotypic screening of isolates for ESBL production showed high resistance 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. Phenotypic screening for β -lactamase production post curing showed reduction of resistance rates by 50%. Varying and high numbers of resistance patterns were identified amongst the isolates ranging from 5 and 15 patterns for *V. cholera* and *Vibrio* sp respectively from river water and 5 and 11 patterns for *V. cholera* and *Vibrio* sp respectively from aquaculture water sources.

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]. According to [11], the presence of antibiotics in the aquatic environment can result in the appearance of resistance among human pathogens forming part of its micro biota. Consequently, bacteria resistant to antibiotics have been found in surface water [12, 13]. According to [14, 15], bacteria with intrinsic or acquired resistance to antibiotics have commonly been found in aquatic environments, where *Pseudomonas*, *Serratia* and *Aeromonas* are commonly identified.

The members of the family *Vibrionaceae* are Gram negative, straight or curved rods which have polar flagella. Most are oxidase positive and all use D-glucose as their sole or primary carbon or energy source. They are mostly aquatic microorganisms widespread in fresh water and sea environment. Several vibrios are important pathogens. *Vibrio cholerae* causes cholera and *V. parahaemolyticus* can cause gastroenteritis in humans following consumption of contaminated sea food [16].

Other vibrios that have been implicated in diseases of human 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 [17].

57 Resistance to different antimicrobials has been detected in environmental isolates of *V.*
58 *cholerae* [18]. While several studies have also identified extended-spectrum β -lactamases
59 from water samples [19], others have reported that the *bla*_{TEM} 1 gene was detected in most
60 of the Gram negative isolates resistant to ampicillin recovered from lakes in Brazil and from
61 waste water treatment plants in China [20, 21]. The *bla*_{TEM} gene was also identified in
62 *Pseudomonas* isolates from river and aquaculture water sources in Nigeria [13].

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64 In view of the above, [22] recommends a continuous surveillance of antimicrobial resistance
65 in *V. cholerae* to detect the emergence of multi-resistant strains. This comes as a result of
66 the fact that *V. cholerae* may acquire antibiotic resistance plasmids from neighbouring
67 microorganisms since different populations of bacteria co-exist in aquatic ecosystems.

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69 This study was therefore undertaken to study multiple antimicrobial resistance in *Vibrio* sp
70 isolated from river and aquaculture water sources and also determine possible role of β -
71 lactamase genes in identified resistance traits.

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74 2. MATERIAL AND METHODS

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76 Sample types, processing and bacterial isolates

77 Water samples for analysis were collected from river water and aquaculture sources in
78 sterile 1L sample containers. Initial bacterial isolation was done by inoculating 1ml of the
79 samples into 5mls of sterile alkaline peptone water (Oxoid) for enrichment of *Vibrio* sp. and
80 tubes were incubated for 24h at $28^{\circ} \pm 2^{\circ}\text{C}$. After incubation, 0.1ml of enriched samples were
81 spread on the dry surfaces of Thio Citrate Bile Salts Sucrose (TCBS) (Lab M) agar plates for
82 the isolation of both sucrose and non-sucrose fermenting *Vibrio* isolates. Isolates were
83 confirmed with API 20 NE (Bio-Mérieux,).

84 Antimicrobial resistant testing and screening for ESBLs

85 Routine antibiograms were determined by the Kirby-Bauer disk diffusion technique on
86 Mueller-Hinton agar (Oxoid, Basingstoke). The antibiotics assayed and their concentrations
87 were ampicillin (10 μg), mezlocillin (75 μg) kanamycin (30 μg), tetracycline (30 μg), carbenicillin
88 (100 μg) and doxycycline (30 μg) (Oxoid, Basingstoke). The results were interpreted
89 according to the criteria established by the [23]. Phenotypic expression of ESBL production
90 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).

Test for beta-lactamase production

Beta-lactamase production was confirmed by the Nitrocefin test [24]. The Nitrocefin test in this study was performed using Nitrocefin discs (Mast discsTM ID) and was used as a confirmatory test for the production of beta lactamase by the *Vibrio* isolates.

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. parahemolyticus* and *V. fulniscus*.

Antimicrobial resistance screening showed that the river water isolates expressed high resistances to 83% of antimicrobials tested, with rates ranging between 81.3 and 97.8%, while resistance was low to moderate at 40.9% for kanamycin (Table 1). For the aquaculture isolates, a similar trend was also noticed, with high resistance rates ranging between 70 and 98.4%. Five of the tested antimicrobials had moderate resistance to kanamycin (Table 2).

A comparative analysis of resistance rates between the vibrios isolated from river water showed that the *Vibrio* sp expressed higher resistances than the *V. cholera* in 4 of the 6 antimicrobials tested. Resistance to carbenicillin and kanamycin was higher in *V. cholerae* (Figure 1). 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 to kanamycin was higher with the *V. cholerae* isolates (Figure 2). Generally however, the river water isolates expressed higher resistances in 5 of the antimicrobials, while the aquaculture isolates expressed a slightly higher resistance for ampicillin (Figure 3). These differences were found significant ($P = .05$) at 95% confidence interval.

Aeromonas sp, non-cholera vibrios and *Plesiomonas shigelloides* 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 [25].

Antibiotic resistant *Vibrio cholerae* strains are increasingly being found worldwide [26, 27, 28, 29, 30]. Analysis of the antimicrobial tests of the *Vibrio* sp isolates 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 to carbenicillin, tetracycline, doxycycline and mezlocillin was equally high at between 81% and 90.3% for both river and aquaculture water samples respectively too.

Resistance to kanamycin was however moderate for both environmental samples. Analysis of multiple resistances shows also that the *Vibrio* isolates from river water expressed multiple resistances (Figure 4). The isolates were resistant to at least two or more antimicrobials with about 30 isolates being resistant to all 5 antimicrobials. More of the *Vibrio cholerae* isolates here expressed multiple resistances than the *Vibrio* sp isolates. About three *V. cholerae* isolates were however resistant to only one drug while no *Vibrio* sp isolate was resistant to only one drug.

Amongst the aquaculture isolates, multiple resistances were found more among the *Vibrio* sp isolates than *Vibrio cholerae* (Figure 5). The *Vibrio* sp was also more abundant in the aquaculture samples than in the river water. The reverse was the case for *V. cholerae* isolates. In similar studies, [18] also found widespread resistance to Ampicillin in his study of aquaculture isolates and [31] equally reported 64% resistance to Ampicillin in their environmental non-O1 *Vibrio* strains. The rate of resistance in the environmental *V. cholerae* isolates correlates with resistance rates of clinical *V. cholerae* O1 strains (83%) of [31] for ampicillin. Continuing he asserts that 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.

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 [32]. Tetracycline- resistant *V. cholerae* O1 strains have been responsible for major epidemics around the world [26, 33].

High levels of multiple resistances were exhibited by the *Vibrio* isolates in this study, which agrees with the studies conducted by [18, 34]. Multiple drug resistance has been reported in a number of studies of fish pathogens and aquaculture environments [35, 36, 37]. *Vibrio* sp isolated from shrimp hatcheries in Indonesia has demonstrated multiple antimicrobial resistance to antimicrobials such as ampicillin, tetracycline, amoxicillin and streptomycin [38] and [39] reported that bacteria resistant to six to ten antimicrobials were common.

In an earlier study, [34] had reported the detection of high frequency (92.9%) of plasmids in their environmental *V. cholerae* isolates. This high frequency also reinforces the idea that R-plasmids are frequently found in environmental strains [40, 41, 42, 43] Bearing in mind that some plasmids can contribute directly to virulence [44] and that *V. cholerae* non-O1 is a widely distributed potential pathogen whose pathogenic mechanisms have not been completely elucidated, the high incidence of R factors among these bacteria may represent a serious health hazard.

In contrast to the above results, none of the strains studied by [31] harboured any plasmids. As different populations of bacteria co-exist in aquatic ecosystems, *V. cholerae* may acquire antibiotic resistance plasmids from neighbouring microorganisms. Therefore continuous surveillance of antimicrobial resistance in *V. cholerae* should be performed to detect the emergence of multi-resistant strains [22].

One *Vibrio* isolate 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 [45, 46]. The enzymes encoded by these genes confer unequivocal resistance to ampicillin, amoxicillin, ticarcillin and carbenicillin ([47, 48].

Analysis of antimicrobial resistance post-curing gave evidence of the resistance associated with the *Vibrio* sp to be mostly plasmid-borne. This is because only one isolate showed any form of post-curing resistance to AMC. Since 1971, transferable ampicillin resistance has been described in *V. cholerae* isolates from Africa, Asia and Latin America [49, 50, 51, 52, 53, 54] Only two plasmidic β -lactamases have however been described [55].

The first, TEM-1 a broad spectrum- β -lactamase was identified in isolates from Asia and Africa [49, 51]. Second, SAR-1, a carbenicillinase with ampicillin hydrolysing activity lower

199 than that of TEM-1, was described in only 2 isolates in Tanzania [51]. In addition to a TEM-1
 200 type β -lactamase, [55] also identified two extended spectrum plasmid-borne enzymes not
 201 related to TEM or SHV type enzymes in *V. cholerae* O1 isolates recovered during several
 202 cholera seasons in Argentina. Resistance to ampicillin and other antibiotics including
 203 tetracycline and carbenicillin were mostly high in the *Vibrio* isolates from both river and
 204 aquaculture water samples.

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207 Table 1: Frequency of antimicrobial resistance of *Vibrio* sp isolated from river water.

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Antibiotics	No (%) of resistant isolates				Total no. of isolates	Total resistance	%
	No. of isolates	<i>Vibrio cholerae</i>	No. of isolates	<i>Vibrio</i> 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)	

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

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225 Table 2: Frequency of antimicrobial resistance of *Vibrio* sp isolated from aquaculture water.

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Antibiotics	No (%) of resistant isolates						Total resistance	%
	No. of isolates	<i>Vibrio</i> sp	No. of isolates	<i>Vibrio cholerae</i>	Total no. of isolates			
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)		

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

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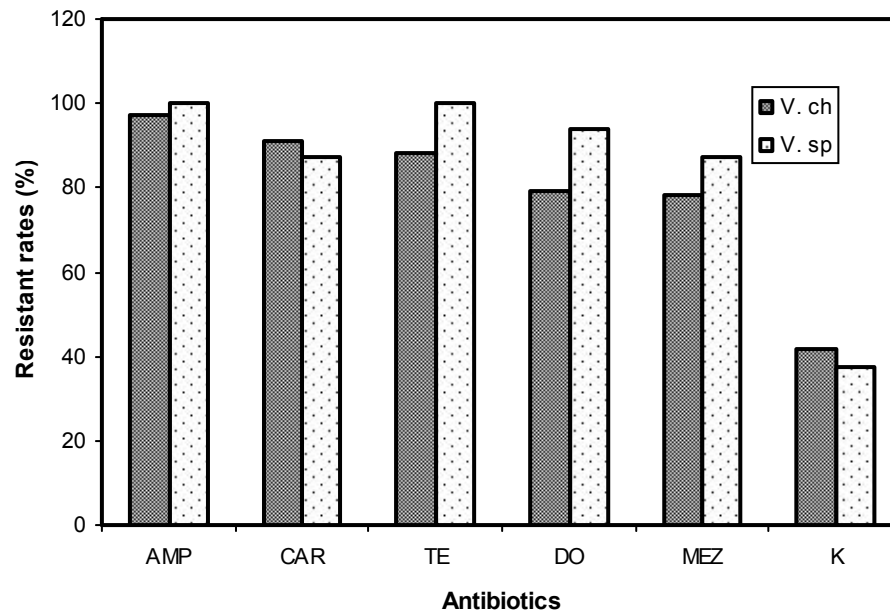
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253 Fig 1: Comparative analysis of rates of resistance between *Vibrio* sp from river water
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256 KEY: V.ch, *Vibrio cholerae*; V. sp, *Vibrio* sp; AMP, Ampicillin; CAR, carbenicillin; TE,
 257 tetracycline; DO, doxycycline; MEZ, mezlocillin; K, kanamycin.

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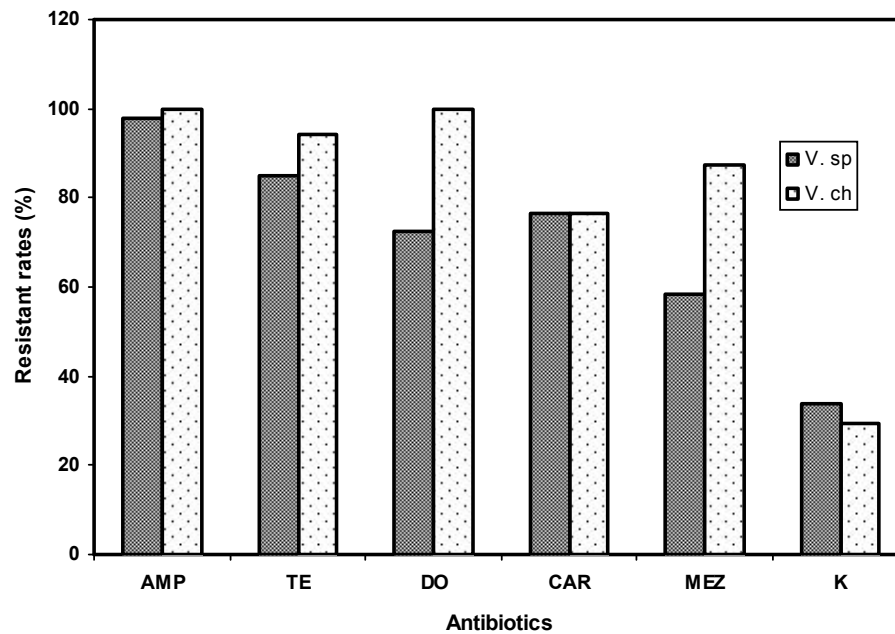
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274 Fig 2: Comparative analysis of rates of resistance between *Vibrio* sp from aquaculture water
275 samples.

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

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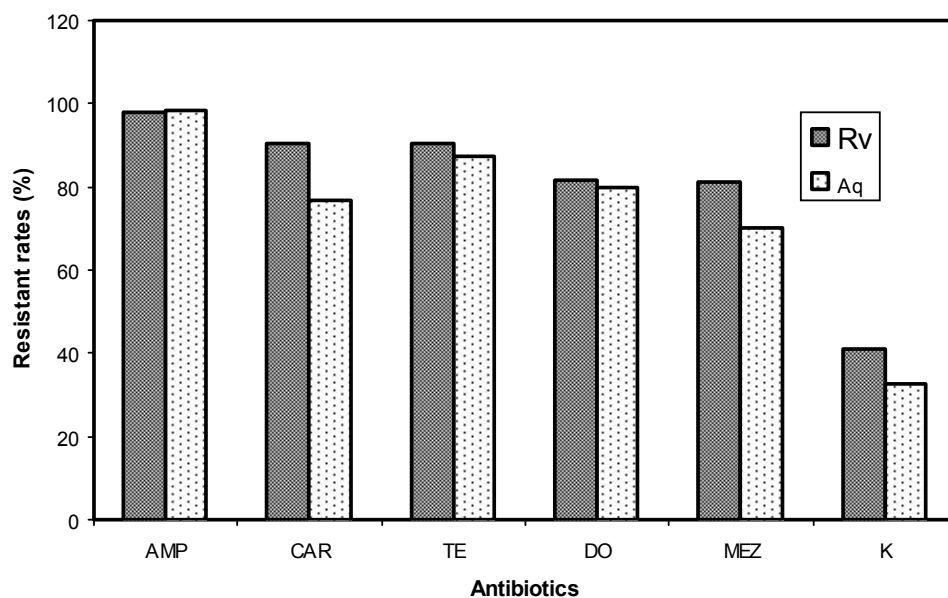
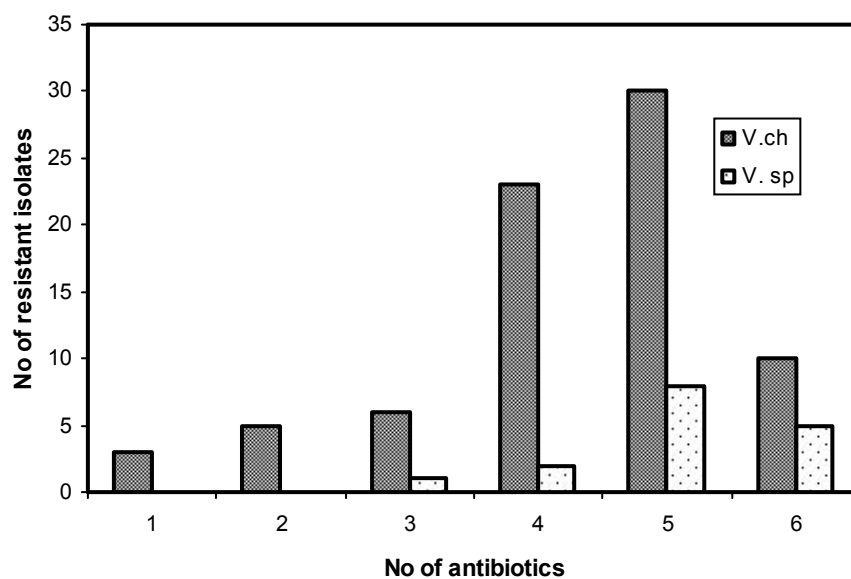


Fig 3: Comparative analysis of rates of resistance between *Vibrio* sp from the river and aquaculture water samples.

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



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312 Fig 4: Multiple resistances in *Vibrio* sp isolated from river water.

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314 Key: *V. ch*, *Vibrio cholerae*; *V. sp*, *Vibrio* sp;

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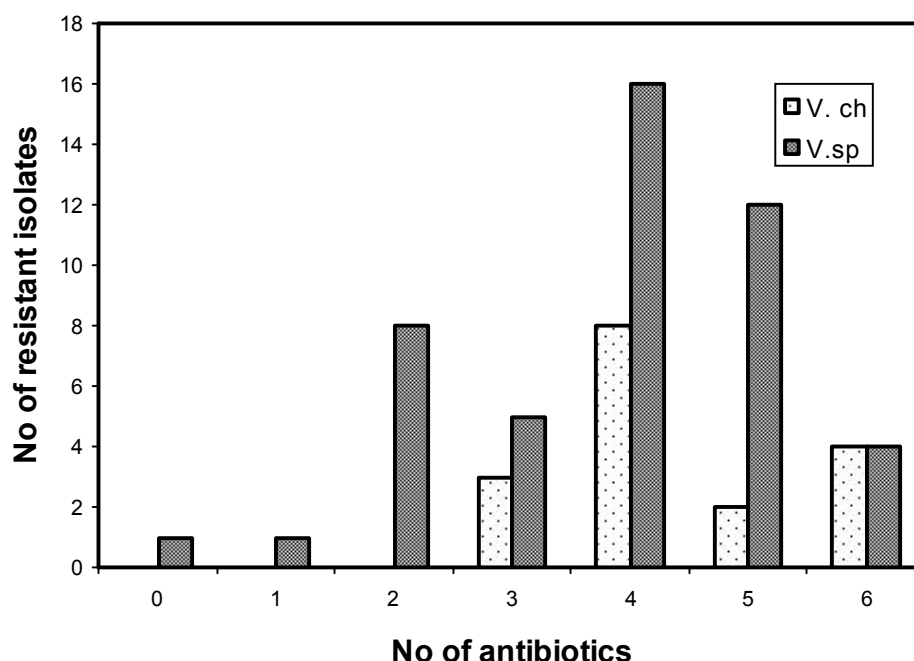


Fig. 5: Multiple resistances in *Vibrio* sp isolated from aquaculture water

4. CONCLUSION

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

335 **COMPETING INTERESTS**

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337 No competing interests exist

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339 **AUTHORS' CONTRIBUTIONS**

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341 Author CIC designed the protocol, carried out the laboratory analyses and wrote the first
342 draft of the manuscript. Authors SNI and GCO supervised the work, analysed the results and
343 edited the manuscript. All the authors collaborated in the final analyses of the study, read
344 and approved the final manuscript.

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