

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

Chikwendu, C. I.*¹, S. N. Ibe² and G. C. Okpokwasili²

¹Department of Microbiology, School of Science, Federal University of Technology, P. M. B. 1529 Owerri, Imo State. ²Department of Microbiology, Faculty of Science, University of Port Harcourt, Choba, Rivers State.

ABSTRACT

Aim: To study multiple antimicrobial resistance in *Vibrio* spp. 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 cholerae*, *V. parahaemolyticus* and *V. vulnificus* 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 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.

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

*Corresponding author

Tel. 2348033186717

E-mail: chinwechikwendu@yahoo.com

1. INTRODUCTION

Bacterial pathogens, once susceptible to antimicrobial agents are becoming increasingly resistant. In addition, hospitals are now facing problems caused by new opportunistic

* Tel.: +xx xx 265xxxxx; fax: +xx aa 462xxxxx.

E-mail address: xyz@abc.com.

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. parahemolyticus* 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].

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

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. MATERIAL AND METHODS

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 Alvan Ikoku 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.

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 $28^{\circ} \pm 2^{\circ}\text{C}$. 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,).

97

98 **Antimicrobial resistant testing and screening for ESβLs**

99 Routine antibiograms were determined by the Kirby-Bauer disk diffusion technique on
100 Mueller-Hinton agar (Oxoid, Basingstoke). The antibiotics assayed and their concentrations
101 were ampicillin (10μg), mezlocillin (75μg) kanamycin (30μg), tetracycline (30μg), carbenicillin
102 (100μg) and doxycycline (30μg) (Oxoid, Basingstoke). The results were interpreted
103 according to the criteria established by the [22]. Phenotypic expression of ESBL production
104 was conducted by the double disk diffusion test with the following antimicrobials: imipenem
105 (10 μg), cefpodoxime (10 μg), cefepime (30 μg), ceftazidime (30 μg), amoxicillin +clavulanic
106 acid (20/10μg) (MAST Diagnostics, England).

107

108 **Test for beta-lactamase production**

109 Beta-lactamase production was confirmed by the Nitrocefin test [23]. The Nitrocefin test in
110 this study was performed using Nitrocefin discs (Mast discs™ ID) and was used as a
111 confirmatory test for the production of beta lactamase by the *Vibrio* isolates.

112

113 **PCR detection of β -lactamase genes**

114 Bacterial chromosomal DNA was used as substrate for the PCR and specific primers for the
115 β-lactamase genes TEM, SHV and CTX were used for the detection of resistance genes
116 (Table 1). PCR was performed in 25 μl of a reaction mixture containing DNA (10-200ng),
117 200 μM of each deoxynucleoside triphosphates (dNTP) (Promega), 1.5 mM MgCl₂, 1X PCR
118 Buffer, 20 pMol (each) of the primers, 1 unit of *Taq* DNA polymerase (Promega) and sterile
119 distilled water. Thermal cycling was conducted in an Eppendorf Master Cycler Gradient at an
120 initial denaturation temperature of 94°C for 5 minutes, followed by 30 amplification cycles of
121 1 minute at 94°C; 1 minute at (50°C for blaTEM, 54°C for blaSHV and 62°C for blaCTX), and
122 1 minute at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The
123 amplification product was separated on 1.5% agarose gel and visualized by staining with
124 ethidium bromide. One hundred (100) bp DNA ladder was used as DNA molecular weight
125 standard.

126

127

128 **Statistical analysis**

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

130

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 to 83% of antimicrobials tested, with the rates ranging between 81.3 and 97.8%, while resistance rates were low to moderate at 40.9% for kanamycin (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 was higher in *V. cholerae* (Figure 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 rates to kanamycin (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 to kanamycin was higher with the *V. cholerae* isolates (Figure 2).

Generally however, the river water isolates expressed higher resistance rates to 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 [27]. Antibiotic resistant *Vibrio cholerae* strains are increasingly being found worldwide [28, 29, 30, 31, 32].

167 Analysis of the antimicrobial tests of the *Vibrio* spp shows that the isolates presented high
168 rates of resistance to ampicillin (97.8% and 98.4%) for both river and aquaculture water
169 samples respectively. Resistance rates to carbenicillin, tetracycline, doxycycline and
170 mezlocillin were equally high at between 81% and 90.3% for both river and aquaculture
171 water samples respectively too. Resistance rates to kanamycin was however moderate for
172 isolates from both environmental samples.

173

174 In similar studies, [18] also found widespread resistance to ampicillin in his study of
175 aquaculture isolates and [33] reported 64% resistance to ampicillin in their environmental
176 non-O1 *Vibrio* strains. The rates of resistance in the environmental *V. cholerae* isolates
177 correlates with resistance rates of clinical *V. cholerae* O1 strains (83%) of [30] for ampicillin.
178 Continuing [33] asserts that one can assume that some of the environmental O1 isolates
179 with antibiotic profiles similar to clinical strains represent strains that have been shed into the
180 environment from clinical cases.

181

182 Total resistance rates to tetracycline at 90.3% and 87.5% for river and aquaculture water
183 isolates respectively is worrisome and of great concern because tetracycline is the antibiotic
184 of choice for the treatment of cholera [34]. Tetracycline- resistant *V. cholerae* O1 strains
185 have been responsible for major epidemics around the world [28, 35].

186

187 All the isolates from both environmental sources were resistant to at least two or more
188 antimicrobials with about 30 isolates being resistant to all 5 antimicrobials. High levels of
189 multiple resistances were exhibited by the *Vibrio* isolates in this study, which agrees with the
190 studies conducted by [18, 36]. Multiple drug resistance has been reported in a number of
191 studies of fish pathogens and aquaculture environments [37, 38, 39]. *Vibrio* spp isolated
192 from shrimp hatcheries in Indonesia has demonstrated multiple antimicrobial resistance to
193 antimicrobials such as ampicillin, tetracycline, amoxicillin and streptomycin [40, 41, 42] and
194 [43] reported that bacteria resistant to six to ten antimicrobials were common.

195

196 One *Vibrio* isolate from aquaculture showed amplification of the *bla*_{TEM} lactamase gene at
197 964bp. According to [30, 44], plasmid mediated *bla*_{TEM} and *bla*_{SHV} are the most common
198 genes encoding beta-lactamases and extended-spectrum beta-lactamases, a major cause of
199 resistance to beta-lactams, and they are increasingly being found in different settings
200 worldwide. The enzymes encoded by these genes confer unequivocal resistance to
201 ampicillin, amoxicillin, ticarcillin and carbenicillin ([45, 46].

202

203 Table 1: The primers used to screen for ESβL genes

204

205

Primer	Gene	Sequence/size (bp)	Reference
757(FP)	blaTEM	5'-GCGGAACCCCTATTTG-3' / 964	24
821(RP)	blaTEM	5'-TCTAAAGTATATATGAGTAAACTTGGTCTGAC-3'/964	
1436(FP)	blaSHV	5'-TTCGCCTGTGTATTATCTCCCTG-3' / 854	25
1437(RP)	blaSHV	5'-TTAGCGTTGCCAGTGYTCG-3' / 854	
1354(FP)	blaCTX	5'-ATGTGCAGYACCAGTAARGTKATGGC-3' / 593	26
1355(RP)	blaCTX	5'-TGGGTRAARTARGTSACCAGAAYCAGCGG-3' / 593	

206

207

208

209

210

211

212

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

214

215

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

216 KEY: AMP, Ampicillin; CAR, carbenicillin; TE, tetracycline; DO, doxycycline; MEZ,

217 mezlocillin; K, kanamycin

218

219

220

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

222

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

223

224 KEY: AMP, Ampicillin; CAR, carbenicillin; TE, tetracycline; DO, doxycycline; MEZ,

225 mezlocillin; K, kanamycin.

226

227

228

229

230

231

232

233

234

235

236

237

238

239

240

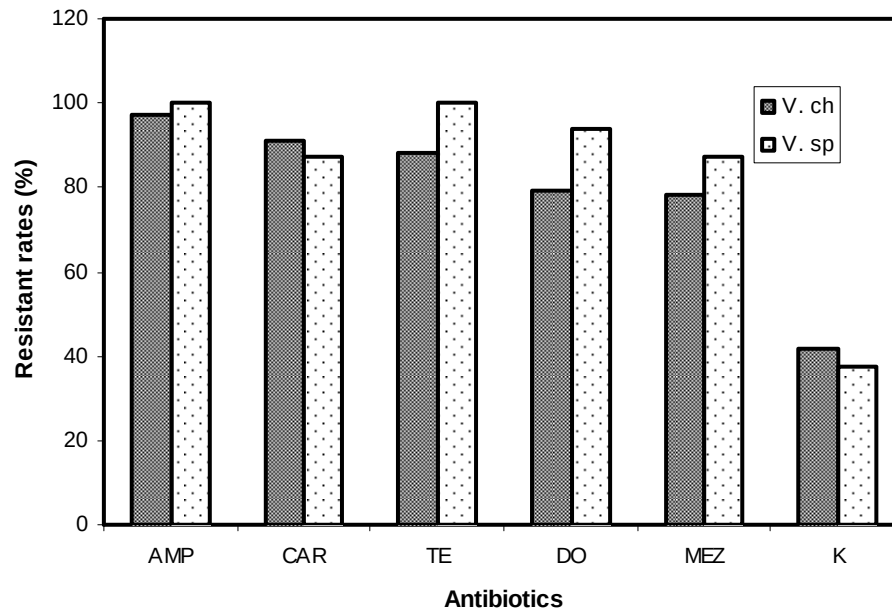
241

242

243

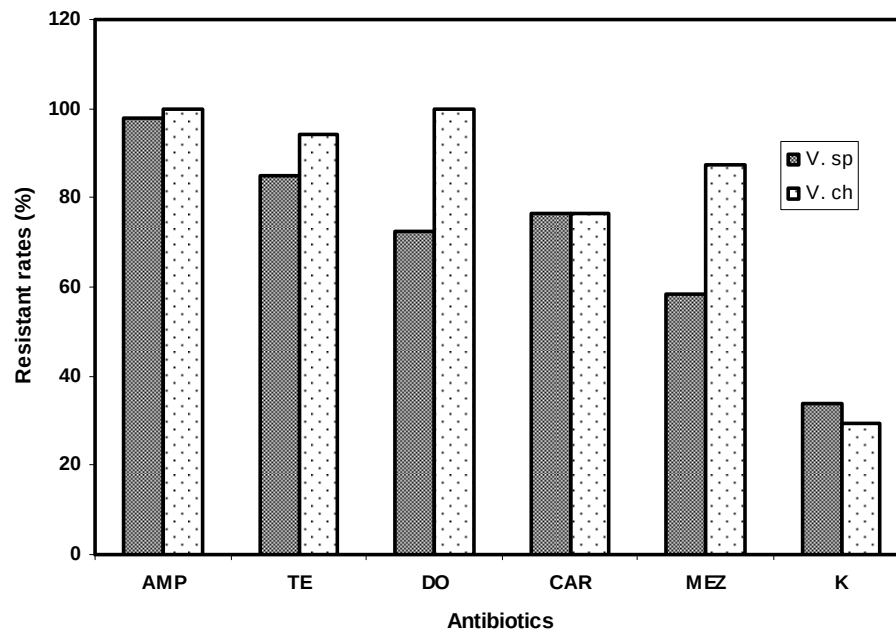
244

245
246
247



248
249 Fig 1: Comparative analysis of rates of resistance between *Vibrio* sp from river water
250 samples.
251
252 KEY: V.ch, *Vibrio cholerae*; V. sp, *Vibrio* sp; AMP, Ampicillin; CAR, carbenicillin; TE,
253 tetracycline; DO, doxycycline; MEZ, mezlocillin; K, kanamycin.

254
255
256
257
258
259
260
261
262
263
264
265



267

268

269

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

272

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

275

276

277

278

279

280

281

282

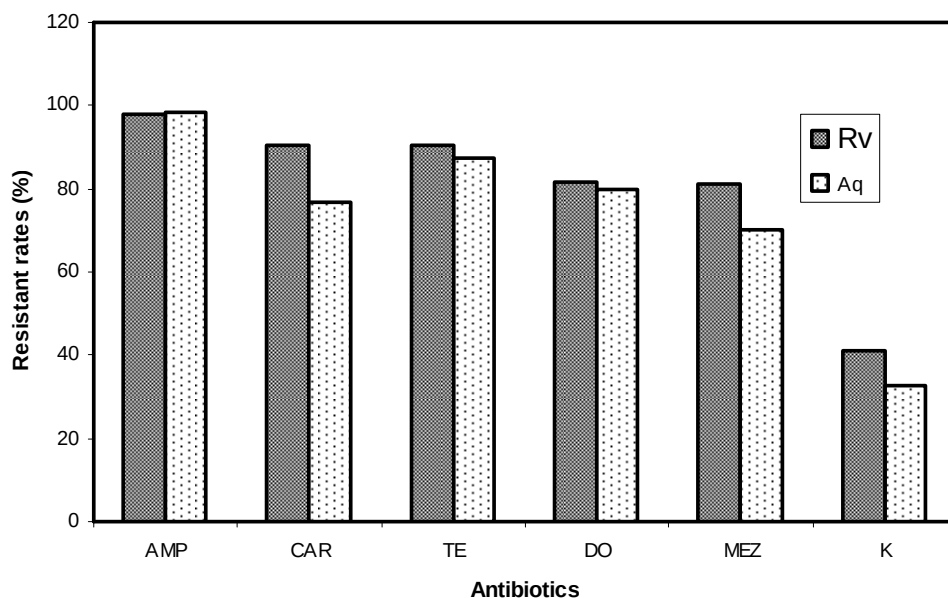


Fig 3: Comparative analysis of rates of resistance between *Vibrio* sp from the river and aquaculture water samples.

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

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 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.

COMPETING INTERESTS

No competing interests exist

AUTHORS' CONTRIBUTIONS

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 the authors collaborated in the final analyses of the study, read and approved the final manuscript.

REFERENCES

1. Quinn JP. Clinical problems posed by multi resistant non-fermenting gram-negative pathogens. Clin. Infect. Dis. 1998; 27(Supl. 1): s117-s124
2. Gaynes R, Edwards JR. Overview of nosocomial infections caused by gram-negative bacilli. Clin. Infect. Dis. 2005; 41: 848-854.
3. Levy SB, Marshall B. Antibacterial resistance worldwide: causes, challenges and responses. Nat. Med. 2004; 10: s122-s129.
4. World Health Organization (WHO). Removing obstacles for healthy development. WHO, Geneva. 1999.
5. Aarestrup F, Ahrens P, Madsen M, Pallesen L, Poulsen R, Westh H. Glycopeptide susceptibility among Danish *Enterococcus faecium* and *E. faecalis* isolates of human and animal origin. Antimicrob. Agents Chemother. 1996; 40:1938-1940.
6. Klare I, Heier H, Claus H, Böhme G, Martin S, Seetmann S, et al. *Enterococcus faecium* strains with vanA-mediated high-level glycopeptide resistance isolated from animal foodstuffs and faecal samples of humans in the community. Microb. Drug Resist. 1995; 1: 265-272.
7. Al-Ghazali MR, Jazrawi SF, Al-Doon ZA. Antibiotic resistance among pollution indicator bacteria isolated from Al-Khair River, Baghdad. Water Res. 1988; 22: 641-644.

- 340 8. Pathak SP, Bhattacharjee JW, Ray PK. Seasonal variation in survival and antibiotic
341 resistance among various bacterial populations in a tropical river. J. Gen. Appl.
342 Microbiol. 1993; 39: 47-56.
343
- 344 9. Halling-Sørensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holton-Lützhøft HC,
345 Jørgensen SF. Occurrence, fate and effects of pharmaceutical substances in the
346 environment- a review. Chemosphere. 1998; 36: 357-393.
347
- 348 10. Goni-Uribe M, Capdepuy V, Arpin C, Raymond N, Caumette P, Quentin C. Impact
349 of an urban effluent on resistance of riverine Enterobacteriaceae and *Aeromonas*
350 spp. Appl. Environ. Microbiol. 2000; 66: 125-132.
351
- 352 11. Angulo FS. Antimicrobial agents in aquaculture: potential impact on health. APUA
353 Newsletter. 2000; 18: 1-6.
354
- 355 12. Schwartz T, Kohnen W, Jansen B, Obst U. Detection of antibiotic resistant bacteria
356 and their resistant genes in waste water, surface water and drinking water biofilms.
357 FEMS Microbial Ecology. 2003; 43: 325-335.
358
- 359 13. Chikwendu CI, Ibe SN, Okpokwasili GC. Detection of bla_{SHV} and bla_{TEM} beta-
360 lactamase genes in multi-resistant *Pseudomonas* isolates from environmental
361 sources. African J. Microbiol. Res. 2011; 5(15): 2067-2074.
362
- 363 14. Aubert D, Girlich D, Naas T, Nagarajan S, Nordmann P. Functional and structural
364 characterization of the genetic environment of an extended-spectrum β -lactamase
365 bla_{VEB} from *Pseudomonas aeruginosa* isolates obtained in India. Antimicrob. Agents
366 Chemother. 2004; 48:3284-3290.
367
- 368 15. Huddleston JR, Zak JC, Jeter RM. Antimicrobial susceptibilities of *Aeromonas* spp.
369 isolated from environmental sources. Appl. Environ. Microbiol. 2006; 72: 7036-7042.
370
- 371 16. Wiley JM, Sherwood LM, Woolverton CJ. Prescott, Harley and Klein's Microbiology
372 7th edn. (International edition). Mc Graw Hill, Boston, 2008; p. 557.
373

- 374 17. Brooks GF, Carrol KC, Butel JS, Morse SA. *Vibrio*, *Campylobacter* and *Helicobacter*
375 and associated bacteria. In: Jawetz, Melnick and Adelberg's Medical Microbiology.
376 24thEdn. McGraw-Hill Com. (LANGE) Inc., New York 2007. Pp. 270-273.
377
- 378 18. Akinbowale OH, Peng H, Barton MD. Antimicrobial resistance in bacteria isolated
379 from aquaculture sources in Australia. *J. Appl. Microbiol.* 2006; 100: 1103-1113.
380
- 381 19. Girlich D, Poiret L, Nordmann P. Diversity of clavulanic acid-inhibited extended-
382 spectrum β -lactamases in *Aeromonas* spp from the Seine River Paris, France.
383 *Antimicrob. Agents Chemother.* 2011; 55: 1256-1261.
384
- 385 20. Li D, Yang V, Hu J, Zhang J, Lui R, Gu X, *et al.* Antibiotic resistance profile in
386 environmental bacteria isolated from penicillin producing waste water treatment
387 plant and the receiving water. *Environ. Microbiol.* 2009; 11: 1506-1517.
388
- 389 21. Pontes DS, Pinheiro FA, Lima-Billencourt CI, Guedes RLM, Cursino L, Barbosa F, *et*
390 *al.* Multiple antimicrobial resistance of Gram negative bacteria from natural
391 oligotrophic lakes under distinct anthropogenic influence in a tropical region.
392 *Microbial Ecology* 2009; 58: 762-772.
393
- 394 22. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for
395 Antimicrobial Susceptibility Testing; Seventeenth Information Supplement, 17th edn.
396 Approved standard M100 – S17. Wayne, PA: Clinical and Laboratory Standards
397 institute. 2007.
398
- 399 23. Cheesbrough M, *District Laboratory Practice in Tropical Countries Part 2*,
400 Cambridge University Press, U.K., 2000. pp. 132-143.
401
- 402 24. Olesen I, Hasman H, Aarestrup FM. Prevalence of β -lactamases among ampicillin
403 resistant *Escherichia coli* and *Salmonella* isolated from food animals in Denmark.
404 *Microb. Drug Resist.* 2004; 10: 334-340.
405
- 406 25. Hasman H, Mevius D, Veldman K, Olesen I, Aarestrup FM. Beta-Lactamases
407 among extended-spectrum β -lactamase (ES β -L)-resistant *Salmonella* from poultry,
408 poultry products and human patients in The Netherlands. *J. Antimicrob. Chemother.*
409 2005; 56: 115-121.

410

411

412 26. Miro E, Navarro F, Mirelis B, Sabaté M, Riviera A, Coll P, Prats G. Prevalence of
413 clinical isolates of *Escherichia coli* producing inhibitor-resistant β -lactamases at a
414 University Hospital in Barcelona, Spain, over a 3-year period. Antimicrob. Agents
415 Chemother. 2002; 46: 3991-3994.

416

417 27. Austin B, Austin DA. Bacterial fish pathogens In: Diseases in Farmed and Wild Fish.
418 Chichester, U.K. Ellis Horwood. 1993.

419

420 28. Chakraborty S, Garg P, Ramamurthy T, Thungapathra M, Gautam JK, Kumar C, *et*
421 *al.* Comparison of antibiogram, virulence genes, ribotypes and DNA fingerprints of
422 *Vibrio cholerae* of matching serogroups isolated from hospitalized diarrhoea cases
423 and from the environment during 1997-1998 in Calcutta. India. J. Med. Microbiol.
424 2001; 50: 879-888.

425

426 29. Dalsgaard A, Forslund A, Bodhidatta L, Serichantalergs O, Pitarangsi C, Pang L, *et*
427 *al.* A high proportion of *Vibrio cholerae* strains isolated from children with diarrhoea
428 in Bangkok, Thailand are multiple antibiotic resistant and belong to heterogenous
429 non-O1, non-O139 O-serotypes. Epidemiol. Infect. 1999; 122: 217-226.

430

431 30. Garg P, Chakraborty S, Basu V, Datta S, Rajendran K, Bhattacharya T, *et al.*
432 Expanding multiple antibiotic resistance among clinical strains of *Vibrio cholera*
433 isolated from 1992-1997 in Calcutta. India. Epidemiol. Infect. 2000; 124: 393-399.

434

435 31. Hofer E, Quintaes BR, Reis EMF, Rodrigues DP, Seki LM, Feitosa IS, *et al.*
436 Emergence of multiple drug resistance in *Vibrio cholerae* isolated from patients with
437 gastroenteritis in Ceara. Brazil. Rev. Soc. Bras. Med. Trop. 1999; 32:151-156.

438

439 32. Ismail H, Smith AM, Sooka A, Keddy KH. Genetic characterization of multidrug –
440 resistant, extended–spectrum– β -lactamase–producing *Vibrio cholerae* O1 outbreak
441 strains, Mpumalanga South Africa. 2008; J. Clin. Microbiol. 2011; 2976-2979

442

443 33. Campos LC, Zahner V, Avelar KES, Alves RM, Pereira DSG, Vital Brazil JM, *et al.*
444 Genetic diversity and antibiotic resistance of clinical and environmental *Vibrio*

445 *cholerae* suggests that many serogroups are reservoirs of resistance. Epidemiol.
 446 Infect. 2004; 132: 985-992.

447

448 34. Mahlu FS, Mmasi PW, Ijumba J. Rapid emergence of El Tor *Vibrio cholerae*
 449 resistant to antimicrobial agents during the first six months of the fourth cholera
 450 epidemic in Tanzania. Lancet. 1979; i: 345-347.

451

452 35. Siddique AK, Zaman K, Banque AH, Akram K, Mutsuddy P, Eusof A, *et al.* Cholera
 453 epidemics in Bangladesh: 1985-1991. J. Diarrhoeal Dis. Res. 1992; 10: 79-86.

454

455 36. Amaro C, Aznar R, Garay E, Alcaida E. R plasmids in environmental *Vibrio cholerae*
 456 non-O1 strains. Appl. Environ. Microbiol. 1998; 54(11): 2771-2776.

457

458 37. McPherson RM, Depaola A, Zywno SR, Moles MI Jnr, Guarino AM. Antibiotic
 459 resistance in gram negative bacteria from cultured catfish and aquaculture ponds.
 460 Aquaculture. 1991; 99: 203-211.

461

462 38. Schmidt AS, Bruun MS, Dalsgaard I, Pedersene K, Larsen JL. Occurrence of
 463 antimicrobial resistance in fish-pathogenic and environmental bacteria associated
 464 with four Danish rainbow trout farms. Appl. Environ. Microbiol. 2000; 66: 4908-4915.

465

466 39. Hatha M, Vivekanandam AA, Joice GJ, Christol GS. Antibiotic resistance pattern of
 467 motile aeromonads from farm raised –fresh water fish. Int. J. Food Microbiol. 2005;
 468 98: 131-134.

469

470 40. Tjahjadi MR, Angka SL, Suwanto A. Isolation and evaluation of marine bacteria for
 471 biocontrol of luminous bacterial disease in tiger shrimp larvae (*Penaeus monodon*,
 472 Fab.). Asia Pac. J. Mol. Biol. Biotechnol. 1994; 2: 347-352.

473

474 41. Rebouças RH, de Sousa OV, Lima AS, Vasconcelos FR, de Carvalho PB, dos
 475 Fernandes Vieira HS. Antimicrobial resistance profile of *Vibrio* species isolated from
 476 marine shrimp environments (*Litopenaeus vannamei*) at Ceafá, Brazil.
 477 Environmental Research. 2011; 21-24.

478

479 42. Mirand CD, Zemelman R. Antimicrobial multi-resistance in bacteria isolated from
 480 freshwater Chilean salmon farms. Sci. Total Environ. 2002; 293: 207-218.

481

482 43. Livermore DM. Beta-lactamases in laboratory and clinical resistance. Clin. Microbiol.
483 Rev. 1995; 8: 557-584.

484

485 44. Pool K. Resistance to beta-lactam antibiotics. Cell Mol. Life Sci. 2004; 61: 2200-
486 2223.

487

488 45. Seetulsingh PS, Hall LM, Livermore DM. Activity of clavulanate combinations
489 against TEM-1 beta-lactamase producing *Escherichia coli* isolates obtained in 1982
490 and 1989. J. Antimicrob. Chemother. 1991; 27: 749-759.

491

492 46. Wu PJ, Shannon K, Phillips I. Effect of hyperproduction of TEM-1 beta-lactamase on
493 in vitro susceptibility of *Escherichia coli* to beta-lactam antibiotics. Antimicrob.
494 Agents Chemother. 1994; 38: 494-498.

495

496

497