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Isolation, Molecular Characterization and Antimicrobial Resistance Patterns of Four Different *Vibrio* Species Isolated from Fresh Water Fishes

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A B S T R A C T

Vibrio species are the major food borne pathogens commonly associated with aquatic food poisonings and lead to food-borne outbreaks. In this present study, out of 105 fresh water fish (*Catlacatla*) samples collected, 87 (82.85%) were found positive for *Vibrio* species. Out of 87, 6 (6.9%), 2 (2.3%), 4 (4.6%) and 3 (3.45%) isolates were found to be *V. parahaemolyticus* and *V. vulnificus*, *V. alginolyticus* and *V. cholerae* respectively by mPCR. The 15 different *Vibrio* species were subjected to antibiogram studies including ESBL detection. Antibiotics like ampicillin, penicillin, gentamycin, amikacin, tetracycline, ceftazidime, streptomycin and co-trimoxazole were used for antibiogram profile. Out of 15 isolates, 5 isolates were found positive for ESBLs by both phenotypic and molecular methods. Out of 5ESBL (only TEM gene) positive isolates, 2 (33.33%), 1 (50%), 1 (25%) and 1 (33.33%) were from *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* and *V. cholera* respectively by mPCR.

Introduction

Members of genus *Vibrio* are defined as gram negative, asporogenous rods that are straight or have a single rigid curve and are motile with a single polar flagellum when grown in liquid medium and they are widely acknowledged as one of the most important waterborne pathogens causing gastrointestinal disorders (Kaysner and De Paola, 2004). *Vibrio* species will be present as contaminants of raw or undercooked sea food (Gopal *et al.*, 2005; Di Pinto *et al.*, 2008; Luan *et al.*, 2008) and consumption of such foods may lead to acute gastroenteritis including diarrhea, headache, vomiting, nausea and fever (Apun *et al.*, 1999; Vongxay *et al.*, 2008; Yang *et al.*, 2008). Igbinosa and Okoh (2008) opined that *Vibrio* spp. are highly prevalent in marine and aquatic environments and is occasionally associated with outbreaks concerning man. The chief route of transmission is by consumption of food and water contaminated with human faeces or sewage, raw fish and

other sea food. Other route of transmission is entry through broken skin when exposed to aquatic environments and marine organisms. In high prevalence areas, chances of cross contamination of foods are also high. In developing countries, massive outbreaks of *V. cholerae* occur mainly via faeco-oral transmission due to poor sanitation (Faruque *et al.,* 1998; Kaper *et al.,* 1995). *V. parahaemolyticus*, *V. vulnificus* and *V. cholera* have been recognized as important food-borne pathogens, which can cause human diseases (Su and Liu, 2007; Zhang and Austin, 2005). *V. alginolyticus* has been categorized as a human pathogen since 1979 and it may result in endophthalmitis, otitis media and food poisoning in infected patients (Ardic and Ozyurt, 2004; Li *et al.,* 2009; Schmidt *et al.,* 1979). The opportunistic pathogen *V. vulnificus* can cause gastroenteritis, septicemia and wound infections, with high fatality rates in immuno-compromised individuals and those with chronic liver disease (Daniels and Shafaie, 2000; Oliver and Kaper, 2001). Antimicrobial resistance is one of the most important public health problems that directly relates to disease management and control (Ansari and Raissy, 2010). Recently higher frequency of multidrug-resistant *Vibrio* has been reported (Ansari and Raisy, 2010; Okoh and Igbinosa, 2010). Production of extended-spectrum β-lactamases (ESBLs) is a significant resistance-mechanism which is a serious threat to the currently available antibiotic armory (Shaikh *et al.,* 2015). So the present study was carried with an objective of studying the prevalence and antibiogram of *Vibrio* speicies of fresh water fish in and around Vijayawada, Andhra Pradesh, India.

Materials and Methods

Standard control and primers

Pure cultures of *Vibrio parahaemolyticus* and *V.vulnificus* obtained from MTCC, Chandigarh were used as positive controls. Oligonucleotide primers were custom synthesized from M/s. Bioserve Biotechnologies Pvt. Ltd. (Hyderabad).

Sample collection

A total of 105 fresh water fish (*Catla catla*) samples were collected from fish markets in and around Vijayawada, Andhra Pradesh. The samples (10grams) were homogenized with 90ml of Alkaline Peptone Water (APW) with 3% NaCl and incubated at 37^{o} C for 24 hours. The enriched cultures were streaked on Thiosulphate Citrate Bile salt Sucrose (TCBS) agar and plates were incubated at 37^{o} C for 24 hours. On TCBS, *V. parahaemolyticus* and *V. vulnificus* produce green coloured colonies where as *V. cholerae* and *V. alginolyticus* produce yellow colored colonies. The respective colonies were further confirmed by biochemical tests and multiplex-PCR (mPCR).

Antibiogram and β-lactamase production

Antibiogram of *Vibrio* species was carried out against 8 different antibiotics like Ampicillin, Gentamycin, Amikacin, Tetracycline, Ceftazidime, Penicillin, Streptomycin and Co-trimoxazole by Kirby Bauer disc diffusion method on Muller Hinton agar (Bauer *et al.,* 1966). Direct colony suspension of each isolate was made in PBS (pH 7.4) and the turbidity was adjusted to 0.5 McFarland units (equivalent to an approximate cell density of 1.5 x 108 CFU/ml). The diameter of inhibition zones was measured and susceptibility patterns of *Vibrio* species were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2014).

Detection of ESBL production was done phenotypically by Phenotypic Screening Test (PST) and Phenotypic Confirmation Test (PCT) as recommended by CLSI (2014)

guidelines.PST was carried out using four indicator β-lactam antibiotics: Cefotaxime (CTX, 30 µg), Ceftazidime (CAZ, 30 µg), Ceftriaxone (CTR, 30 µg) and Aztreonam (AT, 30 µg). Resistance to at least one of the four antibiotics was considered to be positive PST for ESBL production. The positive PST isolates were then subjected to PCT by combination disc method using three pairs of antibiotic discs: ceftazidime (CAZ, 30 µg), ceftazidime plus clavulanic acid (CAC, 30/10 µg), cefotaxime (CTX, 30 µg), cefotaxime plus clavulanic acid (CEC, 30/10 µg) and ceftriaxone (CTR, 30 µg), ceftriaxone plus sulbactam (CIS, 30/10 µg). ESBL production was confirmed when zone diameter around the combination discs was more than or equal to 5 mm when compared to discs containing respective cephalosporin alone (Drieux *et al.,* 2008*).*

Multiplex PCR (mPCR)

DNA was extracted from all the *Vibrio* isolates by using boiling and snap chilling (Swetha *et al.,* 2015) with slight modifications. 1.5ml of enriched broths were taken into micro centrifuge tubes and centrifuged at 8000 rpm for 10 min. Supernatant was discarded, 50µl of nuclease free water was added and placed in boiling water bath at 100^{o} C for 10min. Immediately snap chilled for 10min and centrifuged at 10,000rpm for 5min. The supernatant was taken as template and subjected to different mPCRs and the PCR products were subjected to gel electrophoresis using 1.5% agarose with ethidium bromide as fluorescent dye and visualized using Gel Documentation unit (BIORAD, USA).

mPCR for *Vibrio* species identification

It was done by targeting genus and species specific genes (Table 1). PCR assay was optimized in 25 µl reaction mixture containing 2 µl of DNA template, 12.5 µl of 2x master mix (Go Taq Green Master Mix, Promega), 0.5µl each of forward and reverse primers (10 pmol/µl) and the rest of the volume is made by adding nuclease free water, under standardized cycling conditions: initial denaturation at 94^{0}C for 3 min; 30 cycles of 94^{0}C for 30 s, 60^{0}C for 30 s and 72^{0}C for 120 sec and a final elongation step at 72^{0}C for 10 min.

mPCR for ESBL genes

DNA from all the PCT positive *Vibrio* isolates were subjected to two mPCR assays for detection of ESBL genes (Table 2). PCR assays were optimized in 25 µl reaction mixture containing 2 µl of DNA template, 12.5 µl of 2x master mix (Go Taq Green Master Mix, Promega), 0.5µl each of forward and reverse primers (10 pmol/µl) and the rest of the volume is made by adding nuclease free water, under standardized cycling conditions: initial denaturation at 94^{0}C for 10 min; 30 cycles of 94^{0}C for 40 s, 60^{0}C for 40 s and 72^{0}C for 1 min and a final elongation step at 72^{0}C for 7 min.

Results and Discussion

Out of 105 fresh water fish samples, 87 were found positive for *Vibrio* species. Out of 87 Vibrio species, *V. parahaemolyticus* was found to be more prevalent followed by *V. alginolyticus*, *V. cholerae* and *V. vulnificus* (Fig. 1). In the present study, the overall occurrence rate of *Vibrio* spp. was found to be 82.85% compared to 98.67% and 27.5% as reported by Noorlis *et al.,* (2011) and El-Hady *et al.,* (2015) respectively.

In the present study, we found that 6.9% (6/87) of the *Vibrio* isolates were belonging to *V. parahaemolyticus* whereas high prevalence rates of 24%, 28.6% and 75.9% of *V. parahaemolyticus* were reported by Noorlis *et al.,* (2011), Nelapati and Krishnaiah (2010)

and Anjay *et al.,* (2014) respectively. Adebayo-Tayo *et al.,* (2011) reported a prevalence rate of 2.5% *V. vulnificus* which is in accordance to the present study of 2.3% (2/87) whereas Thampuran and Surendran (1998) reported a high occurrence upto a level of16.6%. In our study, 4.6% (4/87) of isolates were found to be *V. alginolyticus* which was in agreement with Adebayo-Tayo (2011).The occurrence rate of *V. cholerae* was found to be 3.45% (3/87) which was less when compared to 6% reported by Traore *et al.,* (2014). The variations in occurrence of different species of *Vibrio* may be due to level of salinity, geographic, seasonal variations and isolation procedures followed (Kaneko and Clowell, 1975 and Deepanjali *et al.,* 2005).

Table.1 Oligonucleotide primers used for detection of *Vibrio* species by mPCR

Target genes and species	Primer sequence	Product size (bp)	Reference
ompW *V. cholerae*	CACCAAGAAGGTGACTTTATTGTG CGTTAGCAGCAAGTCCCCAT	427	Nandi *et al.,* 2000
gyrB *V. alginolyticus*	GAGAACCCGACAGAAGCGAAG CCTAGTGCGGTGATCAGTGTTG	337	Zhou *et al.,* 2007
collagenase *V. parahaemolyticus*	GAAAGTTGAACATCATCAGCACGA GGTCAGAATCAAACGCCG	271	Di Pinto *et al.,* 2005
vvhA *V. vulnificus*	TTCCAACTTCAAACCGAACTATGA ATTCCAGTCGATGCGAATACGTTG	205	Panicker *et al.,* 2004
16S rRNA (Genus specific)	CCTGGTAGTCCACGCCGTAA CGAATTAAACCACATGCTCCA	168	Wei *et al.,* 2014

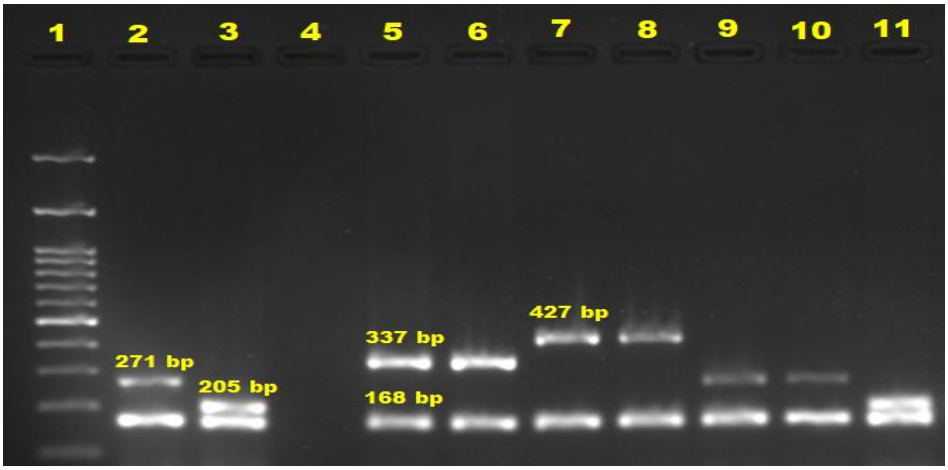
Table.2 Oligonucleotide primers used for detection of ESBLgenes (Dallenne *et al., 2010*)

Primer	β –lactamase (s) targeted	Primer sequence	Amplicon size (bp)
1st mPCR			
blaTEM	TEM-1&2	CATTTCCGTGTCGCCCTTATTC CGTTCATCCATAGTTGCCTGAC	800
blaSHV	SHV-1	AGCCGCTTGAGCAAATTAAAC ATCCCGCAGATAAATCACCAC	713
blaOXA	OXA-1,4&30	GGCACCAGATTCAACTTTCAAG GACCCCAAGTTTCCTGTAAGTG	564
2nd mPCR			
blaCTX-M1	CTX-M-1, CTX-M-3 and CTX-M-15	TTAGGAARTGTGCCGCTGYAb CGATATCGTTGGTGGTRCCATb	688
blaCTX-M2	CTX-M-2	CGTTAACGGCACGATGAC CGATATCGTTGGTGGTRCCATb	404
blaCTX-M9	CTX-M-9 and CTX-M-14	TCAAGCCTGCCGATCTGGT TGATTCTCGCCGCTGAAG	561

Table.3 Details of Antibiotic resistance of *Vibrio* species by phenotypic method

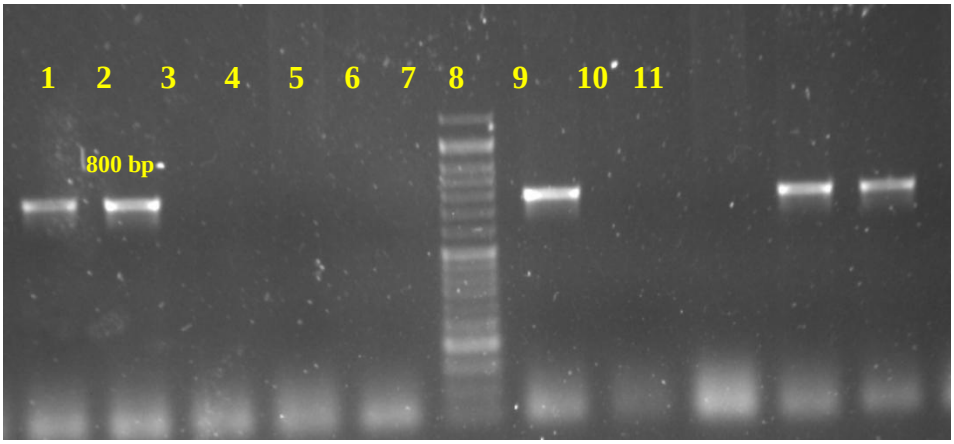
Antibiotics	*V. parahaemolyticus* (6)	*V. vulinificus* (2)	*V. alginolyticus* (4)	*V. cholerae* (3)	Total
Ampicillin (AMP-10µg)	5	2	4	3	14 (93.38%)
Gentamicin (GEN-30µg)	5	2	3	2	12 (80%)
Amikacin (AK-30µg)	4	1	3	2	10 (66.66%)
Tetracycline (TE-30µg)	2	-	2	1	5 (33.33%)
Ceftazidime (CAZ-30µg)	5	2	3	2	12 (80%)
Penicillin (P-10units)	4	1	2	2	9 (60%)
Streptomycin (S-10µg)	1	-	-	-	1 (6.66%)
Co-trimoxazole (COT-25µg)	-	-	-	-	0

Fig.1 Agarose gel electrophoresis of amplified DNA of *Vibrio* species by m-PCR



Lane 1: 100 bp DNA ladder
Lane 2: Positive control of *V. parahaemolyticus* (MTCC 451)
Lane 3: Positive control of *V.vulnificus* (MTCC 1145)
Lane 4: Negative control
Lane 5&6: Sample showing *V.alginolyticus*
Lane 7 & 8: Sample showing *Vibrio cholerae*
Lane 9&10: Sample showing *Vibrio parahaemolyticus*
Lane 11: Sample showing *Vibrio vulnificus*
Lane 2-11 except 4 are showing genus specific gene at 168bp

Fig.2 Agarose Gel Electrophoresis of *bla*$_{TEM}$ gene by m-PCR



Lane 1, 2, 10 and 11: Samples positive for *bla*$_{TEM}$ gene
Lane 3, 4, 5 and 9: Samples negative for *bla*$_{TEM}$gene
Lane 6: 50 bp DNA ladder
Lane 7: Positive control for *bla*$_{TEM}$ gene
Lane 8: Negative control

Out of 15 *Vibrio* isolates, highest resistance was recorded against ampicillin followed by gentamicin, ceftazidime, amikacin, penicillin, tetracycline and streptomycin. All of the isolates were found sensitive to co-trimoxazole (Table 3). The resistance patterns of different *Vibrio* isolates in this study were in accordance with the reports of Raissy *et al.*, (2012), Ansari and Raissy (2010) and Okoh and Igbinosa (2010).

Out of 15 isolates, 5 isolates were found positive for ESBLs i.e. 2 (33.33%), 1 (50%), 1 (25%) and 1 (33.33%) were from *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* and *V. cholerae* respectively by both phenotypic and molecular methods. All 5 isolates showed presence of only *bla*$_{TEM}$ gene and none of the isolates were positive for *bla*$_{OXA}$, *bla*$_{SHV,}$ *bla*$_{CTX-M1,}$ *bla*$_{CTX-M2}$ and *bla*$_{CTX-M9}$genes (Fig. 2). Ismail *et al.*, (2011) reported presence of TEM-63 gene in all the selected 10 isolates of *V. cholera* O1 coinciding with that of ceftazidime MIC of 64 µg/ml. Petroni *et al.*, (2002) analysed 28 isolates of *V. choleae* O1 biotype E1 for presence of ESBLs by susceptibility analysis, isoelectric focusing, PCR-based RFLP and reported that CTX-M type enzymes were identified in 3 isolates and TEM-1 like enzyme in one isolate.

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