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Detection of *Vibrio vulnificus* in water, seafoods and fresh water fish

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Abstract
A study was conducted to record the prevalence and antimicrobial susceptibility of *Vibrio vulnificus* in seafoods and fresh water fish sold in the local markets. Total 300 samples comprised of marine fish (100), fresh water fish (100), marine water (40) and river/lake water (60) were screened for *V. vulnificus* by isolation and PCR. Observed prevalence of *V. vulnificus* was 11% in marine fish, 7% in fresh water fish and 5% in water samples. Isolates were 100% sensitive to chloramphenicol, ciprofloxacin, gentamicin, strepromycin and tetracycline. Few isolates were resistant to rifampicin (56.52%) and amikacin (30.43%). *Class1* integron gene was detected in 39.13% isolates. Sequencing of the *toxR* gene and BLAST analysis revealed identical matches with a number of *V. vulnificus toxR* gene sequences with 85-99% similarity. Detection of *V. vulnificus* in seafood and fresh water fish pose public health risk and alarming in the context of food safety.

Keywords: *Vibrio vulnificus*, seafoods, fresh water fish, class1 integron

Introduction
Vibrio species are Gram negative halophilic bacteria indigenous to marine and estuarine environment. The genus *Vibrio* comprises more than 60 species ubiquitous to aquatic environment and 13 out of them are pathogenic to human. *Vibrio parahemolyticus* and *V. vulnificus* are predominant food borne strains responsible for majority of the seafood related outbreaks worldwide [1]. *V. vulnificus* is an opportunistic human pathogen which may cause gastroenteritis, cellulitis and septicemia. It has been recovered from fish, shell fish, water sediments of a wide range of temperature and salinity [2, 3] Incidence of this bacterium increased considerably during recent year in United States, Japan and Korea [4]. With sustain growth in fish production, improved distribution channels, world fish food supply has grown dramatically in last five decades with an average growth rate of 3.2% per year. World per capita fish supply increased to 18.4 Kg and Asia accounted for 2/3rd of total consumption [5]. India is second largest producer of fish contributing 5.43% of global production. Since *Vibrio* species are one of the important pathogenic microbiota in marine ecosystem, study of their antimicrobial resistance is crucial [6] Development of antimicrobial resistance in fish pathogen and aquatic bacteria is well documented [7] and these resistant determinants can be transmitted to other bacteria of public health significance via contaminated food. Mobile genetic elements like plasmid and transposons has predominant role in the drug resistance transmission [8, 9]
Both, fresh and marine fish are sold daily in local market of the study area and fish is one of the important food commodity locally preferred. Sea foods are procured from coastal market of Konkan and fresh water fish is mainly captured from Nira river. Reports on the occurrence of *Vibrio* species with special reference to *V. vulnificus* in the locally marketed fish found scanty therefore, a study was commenced to detect *V. vulnificus* in marine and fresh water fish including water samples and to reveal their antimicrobial resistance pattern.

2. Materials and Methods
2.1 Sampling
A total of 300 samples comprised 100 each of seafood, fresh water fish and water were collected and analyzed for isolation and identification of *V. vulnificus.* Seafoods were procured from the Mire beach Ratnagiri and local fish market of Shirwal. Fresh water fish were sampled

from Veer dam and local fish markets. Sea water samples were collected from different locations of beach in Ratnagiri and Ganpatipule; whereas locations of fresh water were sourced from Neera river and Veer dam. All the samples were collected and handled in aseptic manner and transported under chilling conditions to the Department of Veterinary Public Health of this institute for further analysis.

2.2 Isolation and identification

It was achieved in two steps *viz.* enrichment in Alkaline Peptone Water (APW pH- 8.5±0.5) and selective plating on Thio Sulphate Citrate Bile Salt Sucrose Agar (TCBS-HiVeg-Selective, HiMedia Laboratories Pvt. Ltd., pH-8.8±0.5) as per the methods of USFDA, 1998. Briefly, a 25 gm sample of fish was enriched with 225 ml APW and incubated at 37 ℃ for 24 h. Loopful of enriched culture was streaked on TCBS agar and plates were incubated at 37 ℃ for 24 h. Plates showing typical yellow and greenish blue colonies were picked, subcultured again on TCBS agar and presumptive confirmation was done by biochemical tests.

2.3 Molecular detection

Presumptive *Vibrio* isolates were identified at species level by Polymerase Chain Reaction (PCR) designed by Baur and Rorvik [10] PCR primers used to amplify *toxR* gene of *V. vulnificus* were forward (5'-3') - GASTTTGTTTGGCGYGARCAAGGTT and reverse (5'-3') - AACGGAACTTAGACTCCGAC. The genomic DNA was extracted from the pure colonies by boiling and snap chilling in crushed ice. PCR was performed in 25µL volume containing 12.5µL PCR master mix, one micro liter each of forward and reverse primer, two micro liter DNA template and 8.5µL nuclease free water to make final volume. PCR conditions were set in Applied Biosystems Veriti 96 thermal cycler as: initial denaturation (95 ℃/4 min) followed by 25 cycles of denaturation (95 ℃/30 s), annealing (55 ℃/30 s), extension (72 ℃/30 s) and final extension (72 ℃/7 min). Amplified products were separated by gel electrophoresis on 1.2% agarose stained with ethidium bromide and images were captured in gel documentation system (G-BOX F3 Syngene).

2.4 Antimicrobial susceptibility

It was performed by disk diffusion method described earlier [11]. The clinical breakpoints were defined according to the recommendations of Clinical and Laboratory Standard Institute [12]. Antimicrobials used in the study were amikacin (30 mcg/unit), ampicillin (10 mcg/unit), chloramphenicol (30 mcg/unit), ciprofloxacin (5 mcg/unit), gentamicin (10 mcg/unit), tetracycline (10 mcg/unit), rifampicin (5 mcg/unit) and streptomycin (10 mcg/unit). Briefly, overnight grown cultures in Brain Heart Infusion (BHI) broth were spread on Mueller Hinton Agar (MHA), antimicrobial disks were placed and plates were incubated at 37 ℃ for 24 h. Results were recorded as sensitive, intermediate and resistant as per the manufacturers instructions (HiMedia Laboratories Pvt. Ltd., Mumbai, India). Multiple Antibiotic Resistance (MAR) index for individual isolates was also calculated [13]

2.5 Detection of *class1* integron gene

It was detected by PCR method described by Igbinosa and Okoh [14] Oligonucleotide sequences used for amplification of *class1* integron gene were forward (5'-3') GGCATCCAAGCAGCAAG and reverse (5'-3') GGCATCCAAGCAGCAAG. PCR was performed in 25µL volume containing 12.5µL PCR master mix, one micro liter each of forward and reverse primer, three micro liter DNA template and 7.5µL nuclease free water to make final volume. PCR conditions were set as: initial denaturation (94 ℃/2min) followed by 30 cycles of denaturation (95 ℃/45 s), annealing (56 ℃/60 s), extension (72 ℃/90 s) and final extension (72 ℃/10 min). Amplified products were separated by gel electrophoresis on 1.2% agarose stained with ethidium bromide (0.5 mg/L) and images were captured in gel documentation system (G-BOX F3 Syngene).

2.6 Neighboring distance tree generation

The evolutionary history was inferred using the Neighbor-Joining method showing the phylogenetic interrelationships of *V. vulnificus* strains with related *Vibrio* sp. based on toxR gene nucleotide sequences [15] The optimal tree with the sum of branch length = 8.66125948 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [16] and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1137 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [17].

3. Results and Discussion

The bacterium *V. vulnificus* is unique in its characteristics as far as its survival and distribution in aquatic ecosystem is concerned. This bacterium may be isolated from range of aquatic ecosystems and wide variation in temperature and salinity [18] *V. vulnificus* may cause septicemia and wound infections in human and its principal route of transmission is sea foods. Several studies documented the isolation and characterization of *V. vulnificus* from seafood and marine environment including reports from India [19-21], however, reports on its isolation from fresh water ecosystem is poorly studied.

In this study, the isolation of *V. vulnificus* from marine as well as fresh water fish and water sample was attempted. Out of 300 samples, 23 *V. vulnificus* were isolated with overall prevalence of 7.66%. Seven isolates were from fresh water fish, eleven isolates were from seafoods, two isolates were from fresh water samples and three isolates were from sea water samples collected from different sites (table 1). Samples collected and processed for isolation were muscle part of fish, whereas, marine water samples were procured from different locations of fish landing area and fresh water sampled from different locations of lake and river.

V. vulnificus is a comparatively less studies food borne pathogen than *V. parahemolyticus.* Limited reports are available from India on isolation and characterization of *V. vulnificus.* It has been isolated from marine ecosystem, seafoods from coastal regions [22, 23] Recently, *V. vulnificus* has been isolated from fresh water fish catla [24]. Detection of *V. vulnificus* in fresh water fish and fresh water samples is indicative of exploring the role of fresh water ecosystem in survival and distribution of *V. vulnificus*. Isolation of *V. vulnificus* biotype 1 and 2 from sea water and shellfish and its co-relation to water temperature and salinity from Danish marine environment was reported by Hoi *et al.* [18] Their findings suggested marine ecosystem as an important

reservoir of *V. vulnificus*.

Recently, virulent biotype 1 *V. vulnificus* has been isolated from clam samples collected from local fish market and estuary in Manglore [23] In another study from India, eight percent prevalence of *V. vulnificus* in fresh water Catla fish was recorded [24]. An extensive investigation on abundance of *V. vulnificus* in two Indian estuaries revealed influence of temperature and salinity on the presence and densities of *V. vulnificus* in oysters [20]

It was observed that *V. vulnificus* isolated from different samples in the present study were 100% susceptible to chloramphenicol, ciprofloxacin, gentamicin, streptomycin and tetracycline. Resistance was recorded for ampicillin (100%), amikacin (65.21%) and rifampicin (39.13%). Ampicillin resistance *Vibrio* species have been isolated frequently from aquaculture and seafoods [21, 25] Amikacin resistant pathogenic *V. vulnificus* were recently isolated from shrimp [26]. MAR index was not recorded since percentage of multidrug resistant *V. vulnificus* was very less. Generally MAR index >0.2 is considered to have originated from a high risk source of contamination [27] In contrary to these observations, multiple drug resistant *Vibrio* species including *V. vulnificus* were isolated from fish pond facilities of Benin city, Nigeria [28]. *V. vulnificus* isolates of this study were also 100% susceptible to chloramphenicol, ciprofloxacin, gentamicin, streptomycin and tetracycline. In contrary, *V. vulnificus* strains resistant to streptomycin and tetracycline were isolated from wastewater effluents [29]. Choramphenicol and streptomycin intermediate and gentamicin sensitive *V. vulnificus* were also recovered from recreational and commercial areas of Chesapeake bay and Maryland Coastal bays by Shaw *et al.* [22]

Class 1 integrons are usually reported to contain antibiotic-resistant gene cassettes and are related with other mobile elements like plasmids, which could contribute to the dissemination of resistance genes [14]. *Class-1 integron* gene was detected in 39.13% *V. vulnificus* isolates. Maximum isolates were from seafoods and marine water samples. Class1 integrons containing various resistance gene cassettes which are distributed among different *Vibrio* species are documented earlier [30, 31]

Table 1: Overall occurrence of *V. vulnificus* in different samples collected

Sr. No.	Source	No. of samples	No. of *V. vulnificus* isolates	Occurrence (%)
1	Seafood samples	100	11	11
2	Fresh water fish samples	100	7	7
3	Lake /river water samples	40	2	5
4	Seawater samples	60	3	5
Total		300	23	7.66

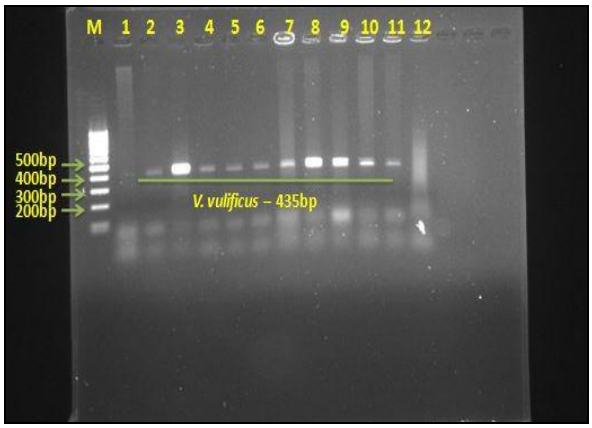

Fig 1: Detection of *V. vulnificus toxR* gene by PCR M: 100 bp DNA ladder; Lane 2 - 11: *V. vulnificus* isolates showing amplification of toxR gene (435 bp)

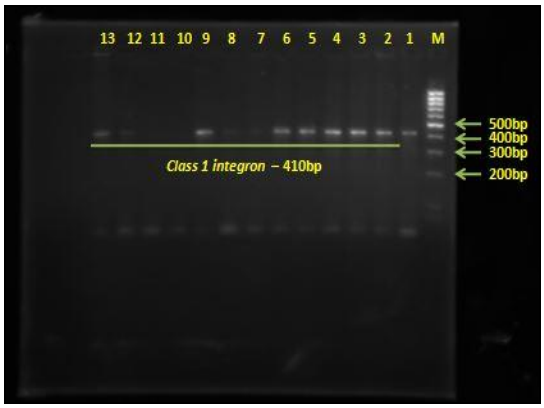

Fig 2: Detection of *Clss 1 integron* gene in *V. vulnificus* isolates by PCR M: 100 bp DNA ladder (100bp); Lane 1- 6; 9 and 13 showing amplification of *Clss 1 integron* gene (410 bp)

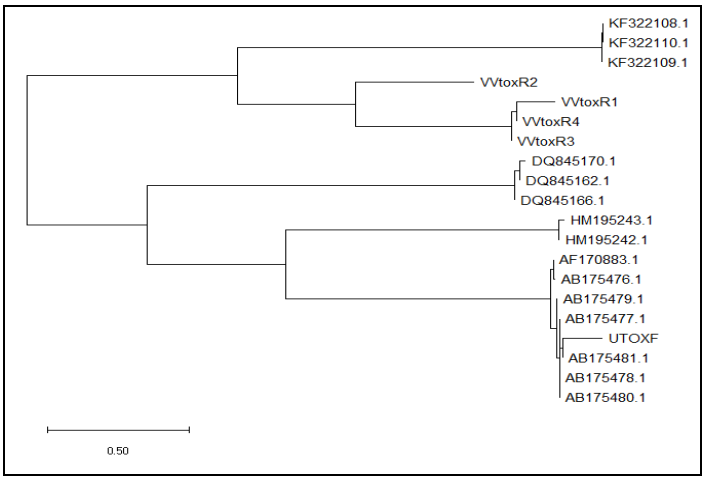

Fig 3: Neighbour joining tree of *V. vulnificus* toxR gene

4. Conclusion

This preliminary study indicated that marine environment could be a potential pool for acquiring antimicrobial resistance genes. Isolates formed separate clad (VVtoxR1, R2, R3 and R4) stating their evolution at different period. Present findings highlighted the importance of fresh water and marine foods in the harboring and possible transmission of *Vibrio vulnificus* which is one of the potential bacterial food borne pathogens associate with fish and fish products.

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