

## Incidence of multidrug resistant *Vibrio parahaemolyticus* isolated from Ponnani, South India

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### ABSTRACT

**Background and Objectives:** The prevalence of *Vibrio parahaemolyticus* has been reported from Ponnani earlier, however incidence of multidrug resistant strains have been encountered recently in clinical laboratories. The source for such strains and their presence in this major fish landing centre has been investigated.

**Materials and Methods:** Antibiotic sensitivity tests on isolates of *V. parahaemolyticus* isolated from three different substrates were conducted following disc diffusion method.

**Results:** Populations of *V. parahaemolyticus* (cfu/ml) were relatively high in sediment samples ( $7.67 \pm 2.08$ ), compared to shrimp ( $5.33 \pm 1.53$ ) and water samples ( $3.67 \pm 1.15$ ). *V. parahaemolyticus* isolated from water showed relatively higher antibiotic resistance pattern compared to other two groups. The highest incidence of antibiotic resistance was recorded against cephalothin and nitrofurantoinine; the lowest was against tobramycin, piperacillin and amikacin. Maximum multiple drug resistant (MDR) strains were encountered from water samples followed by shrimps.

**Conclusion:** Results emerging from the present study clearly showed that Ponnani has a fairly good population of antibiotic resistant strains of *V. parahaemolyticus*. The present study provides an insight on the microbial population of *V. parahaemolyticus* in Ponnani harbour and warrants the need to develop control measures to reduce incidences of post-harvest contamination of seafood.

**Keywords:** *Vibrio parahaemolyticus*, Antibiotic resistance, Ponnani

### INTRODUCTION

India is the second largest fish producing nation in the world, exporting to 127 countries. It has the potential to grow further in view of the growing demand in export to European Union (EU), United States (US), China and Middle East. However, in the past, export of seafood from India has been banned due to lack of quality especially owing to the stringent enforcement of quality standards by the importing countries. In 1997, the EU banned Indian

seafood, citing lack of hygienic and phyto-sanitary measures in the industry. Since then Indian seafood has made a sea change in its quality assurance. Hence seafood quality presently is most pivotal aspect of seafood trade. The main impediments in the proper utilization of the seafood are its high perishability and health risk due to contaminated pathogens. Microbial contamination is a major problem as evidenced from the reports on a worldwide basis (1-8). Microbial hazards include many bacterial pathogens associated with food born disease. These are broadly classified into two major groups: those that are naturally present in the environment which is indigenous to the food at the time of harvesting and those that get entry in to fish during various stages of handling or those introduced in to the environment from external sources. The bacterial quality of a processed food material depends, to a great extent,

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on the sanitary conditionings of the processing unit. One major group of bacteria that can cause seafood contamination is the genus vibrio. Among halophilic vibrios *Vibrio parahaemolyticus* and *V. vulnificus* are the major pathogens (9). *V. parahaemolyticus* is a Gram negative rod, exhibiting pleomorphism, ranging from straight, slightly curved, and coccoid to swollen forms. It is of marine origin and can be found in seawater, sediments, plankton, fin fishes and shell fishes of coastal and estuarine environments. In India, the occurrence of *V. parahaemolyticus* in fish and aquatic environments has been reported by several workers and the incidence in fresh, marine and brackish water fish varied from 35-55%. The organism can grow at pH values ranging from 4.8-11. It does not cause any marked organoleptic changes to sea foods, even at infectious concentration. In spite of large infectious dose of  $10^7$  to  $10^8$  organisms, the short generation time enables the strains to multiply rapidly at ambient temperatures, this results in sufficient bacteria in seafood to cause disease (10). *V. parahaemolyticus* is very heat sensitive and can be inactivated by mild heat. In man *V. parahaemolyticus* usually causes diarrhea, occasionally dysentery or gastroenteritis of sudden onset varying from mild to severe. Outbreaks of *V. parahaemolyticus* infections usually occur in summer (11).

Ponnani is one of the leading fish landing centers in South India. Annually over 25,000 tons of fin and shell fishes are landed here. One of the major contributors of the fish catch is the "mini trawls" that specializes in shallow water fishing. In spite of rich history in seafood landing, Ponnani is devoid of a seafood processing plant. Incidences of pathogenicity of *E. coli* and *V. parahaemolyticus* (12, 13) in the past has been the major impediments in transforming Ponnani as a seafood processing hub. However, with the commissioning of Ponnani harbor in 2011, the area is all set to increase the fishing and processing capacity. In this regard, a study was envisaged to delineate the cause of prevalence of pathogenic forms of *V. parahaemolyticus* with regard to its habitat and also to investigate whether the newly constructed harbor could ensure quality seafood in the region. Hence the study is the preliminary attempt to isolate and biochemically characterize *V. parahaemolyticus* from various substrate and also analyses the antibiotic resistant pattern of the isolates. Although there have been studies on the pathogenicity of marine vibrios from the coastal waters of Ponnani (13), no attempt

has since then been made to study the occurrence of *V. parahaemolyticus* from the sediment and shrimp samples entering the region. Under the above backdrop the study was conducted to isolate *V. parahaemolyticus* from sea water, sediment and shrimps of Ponnani harbor and to study the antibiotic resistance pattern for selected group of antibiotics among the isolates selected from different substrates.

## MATERIALS AND METHODS

**Study area.** Samples were collected from newly constructed Ponnani fishing harbor ( $10^{\circ} 47' N$  and  $75^{\circ} 57' E$ ). The study was initiated within one year of commissioning of the Ponnani harbor. Generally, fishing activity in Ponnani is concentrated from Puthuponnani to Ponnani bar moth, which includes 4 Km of coastal area. The samples for the present study were collected from relatively new area of the Ponnani landing centre.

**Sampling.** Adequate samples of water, sediment and shrimps were collected on a monthly basis from prefixed locations of the harbor. Since the availability of *V. parahaemolyticus* was reported to be more during summer seasons, three successive monthly samplings were carried out during the months of March to May.

**Water sample.** About 100 ml of the water were collected in sterile conical flask by using a sterile measuring cylinder. One milliliter of water sample was serially diluted in autoclaved normal saline, plated and processed for analysis.

**Sediment sample.** Nearly 50 g of sediment were aseptically scooped out from the surface in a sterile pot. Approximately 10g of the sediment was carefully transferred into a 100 ml sterile beaker and 50 ml of autoclaved distilled water was added to it and thoroughly mixed using a sterile glass rod. The sediment was allowed to settle and the supernatant were transferred to test tubes for serial dilution in autoclaved seawater, plated, and processed for further microbiological analysis.

**Shrimp sample.** About 50 g of shrimp landing at the harbor were directly transferred into a sterile container. Whole tissue were separately taken aseptically from the shrimp and placed in individual sterile test tubes and homogenized in 3 ml autoclaved

normal saline using sterile glass rod. The samples were serially diluted in autoclaved seawater, plated, and processed for further microbiological analysis.

**Direct plate count.** Enumeration of colony forming units of *V. parahaemolyticus* per milliliter (cfu/ml) of water was carried out through direct plating onto tryptone salt agar (T<sub>1</sub>N<sub>3</sub> agar) (1% tryptone, 3% NaCl, 2% agar) medium.

**Isolation and identification.** About 25 g of the sample was blended with 225 ml of Alkaline Peptone Water (APW) and transferred aseptically in to a sterile 500 ml conical flask before incubating at 36 ± 1°C for 16-18 hours. After enrichment, culture from Alkaline peptone water were then streaked on pre-set TCBS (Thiosulphate Citrate Bile salt Sucrose) Agar plates and incubated at 36 ± 1°C for 24 hours. The colonies were then examined after 24 hours of incubation. Colonies that formed blue green color with blue or green centre on TCBS agar indicated the presence of *Vibrio spp.* Typical colonies that showed presence of *Vibrio spp.* on TCBS agar plates were then picked and inoculated in to Nutrient agar slant and incubated for 36 ± 1°C for 24 hours for further analysis.

**Inoculation in to TSI (Triple Sugar Iron) agar and KIA (Kliger Iron Agar).** Colonies from Nutrient agar slants were inoculated on to TSI & KIA slant by stabbing butt and streaking slant. This was then incubated at 36 ± 1°C for 24 hours. Later the slants were observed for typical reactions of *V. parahaemolyticus* i.e., presence alkaline slant (red) and acid butt (yellow) without the production of H<sub>2</sub>S were observed in both TSI and KIA. Results indicated the typical reactions of *V. parahaemolyticus*.

**Biochemical tests.** Cultures giving typical reactions specific to *V. parahaemolyticus* were confirmed following various biochemical tests such as Oxidase test, Gram staining, Motility test, Indole production test, Voges Proskauer test, Fermentation study of sugars for glucose, arabinose, manitol, D-cellobios etc, and Hugh-Leifson test.

**Salt tolerance studies (Halophilicity).** Growth in presence of 0%, 3%, 6%, 8% & 10% NaCl in tryptone broth were studied. To about 5 ml of tryptone broth one loopful of culture was introduced and incubated for 24 hours. Positive tubes have visible growth as

turbidity. There is no turbidity in the tubes containing 0% and 10% salt.

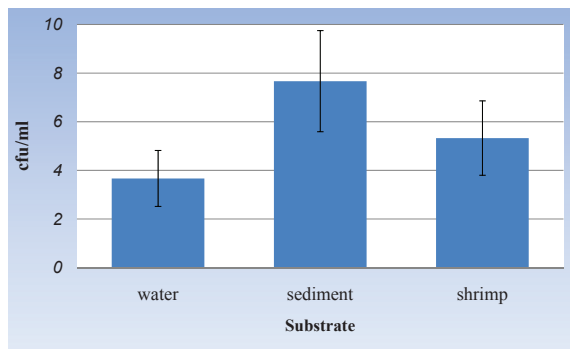
**Antibiotic sensitivity test (Disc diffusion test).** Bacterial isolates were tested for antimicrobial sensitivity using the disc diffusion method. The results were interpreted based on the recommendations of National Committee for Clinical Laboratory Standards for Antimicrobial Susceptibility tests (14). Around 24 hour old culture from the alkaline peptone water were spread as thick straight lines across the surface of pre-set & dried plates of MHA (Hi-media laboratories, Mumbai) using sterile cotton swabs, which were then left to dry for 10 minutes before placing the antimicrobial sensitivity discs. A total of 12 antibiotic discs which includes amikacin (Ak-30 µg), amoxicillin (Ax, 10 Ng), cefazolin (Cz- 30 µg), cephalothin (Kf- 30 µg), chloramphenicol (C-30 µg), ciprofloxacin (Cf-5 µg), nalidixic acid (Na- 30 µg), nitrofurantoinine (F - 100 µg), cefotaxime (Ctx- 30 µg), pefloxacin (Pf - 5 µg), piperacillin (Pr1-100 µg) and tobramycine (Tob-10 µg) were employed. After incubation, the zone of inhibition was measured and compared with zone diameter interpretative chart to determine the sensitivity of the isolates to the antibiotics. Antibiotic sensitivity test were done for each triplicate samples.

**Antibiotic resistance index.** Antibacterial resistance index (ARI) of each samples was determined using the formula  $ARI = y/nx$ , where y was the actual number of resistance determinants recorded in a population of size n and x was the total number of antibacterial tested lot in the sensitivity test (15).

**Statistical analysis.** Antibiotic resistance pattern in *V. parahaemolyticus* from different substrates were tabulated. Means of antibiotic resistance pattern were compared using one-way analysis of variance (ANOVA) using statistical software (SPSS 17.0 for Windows). Post hoc test were carried out using Duncan multiple range tests, if they were significant.

## RESULTS

Results of direct plating to T<sub>1</sub>N<sub>3</sub> Agar to estimate the colony forming units per milliliter of sample revealed that the population of *V. parahaemolyticus* were relatively high in sediment samples (7.67 ± 2.08), followed by shrimp (5.33 ± 1.53) and least in



**Fig. 1.** Population of *V. parahaemolyticus* from different substrates.

water sample ( $3.67 \pm 1.15$ ) (Fig. 1). Results indicate a heavy population of *V. parahaemolyticus* in bottom sediment and this may be due to dumping of coastal sediments in that particular region for construction of the harbor.

After the identification of isolates by using the biochemical tests the isolates were used for antibiotic sensitivity studies. Results presented herewith are means of triplicate samples collected for each substrate. While analyzing the zone of clearance it was observed that the pattern of antibiotic resistance varied between isolates from different substrates. Table 1 shows the comparison of mean values of Antibiotic resistance profile from different substrates. Results indicate that the pattern of resistance is highly significant ( $P < 0.01$ ) for cefotaxime, amoxicillin, ciprofloxacin and pefloracin, while it was moderately significant ( $P < 0.05$ ) for chloramphenicol.

On further analysis using Duncan's Multiple Range Test (DMRT) to delineate the groups based on their antibiotic resistance it was seen that all the five above mentioned antibiotics varied in their action against isolates from different substrates (Table 2). This indicates that the strains of *V. parahaemolyticus* available in the three substrates are different and hence warrant the need for serotyping and determination of pathogenicity through more advanced techniques.

**Means with similar letter as superscript are homogeneous.** Results of Antibiotic resistance pattern in *V. parahaemolyticus* from different substrates is depicted in Table 3. Generally, it could be seen that maximum (16) sensitivity to antibiotics were recorded among sediment isolates, while only five antibiotics were sensitive to isolates collected from water (Table 3). Results therefore points out to the fact that though in terms of number, the

population of *V. parahaemolyticus* was relatively small in water, when it came to diversity, especially antibiotic resistant strains their number was high. This was further confirmed with results of multiple antibiotic resistant strains which was highest (15) for water followed by shrimps (14). While calculating the Antibiotic resistance index it could clearly be state that strains of *V. paahaemolyticus* in water had better antibiotic resistance capacity (0.028) than shrimp (0.023) or sediment (0.021) samples.

## DISCUSSION

Members of the genus *Vibrio* (Vibrionaceae) are Gram negative, usually motile rods having a facultative fermentative metabolism (15). They are generally able to grow on marine agar and selective medium Thiosulfate Citrate Bile salt Sucrose Agar (TCBS) and are mostly oxidase positive. The present study attempted to isolate and biochemically characterizes *V. parahaemolyticus* from various substrate such as seawater, sediment and freshly caught shrimp that landed in Ponnani harbour. *V. parahaemolyticus* is a food born pathogenic bacteria which could be widely found in coastal areas, salt lakes and seafoods, it could cause large scale food poisoning (17). The microbial status of seafood after catch is closely related to environmental conditions and the microbiological quality of the water (18). These factors includes water temperature, salt content, distance between localization of catch and polluted areas, especially those containing human and animal feces, natural occurrence of the bacteria in the water, sediment, injection of food by fish, methods of catch and chilling and post harvest and processing conditions. This bacterium is a potential hazard in shell fish also (17). In the present study a notable proportion of *V. parahaemolyticus* was recorded from sediments as compared to water and shrimp. This indicates the sediments of Ponnani harbor act as sinks for this species. Their higher number may also be due to the land filling that occurred during the construction of new Ponnani harbor. Most of the soil used for this filling was dredged from nearly coastal regions as a result there was a huge deposition of bacterial laden soil in the region. Generally sediment provides better microenvironment than water and thus rich flora can flourish. It has been reported by earlier authors that the flora of the sediment was three times (19) and ten times (20) higher than the water. This high value might

**Table 1.** Comparison of means on Antibiotic resistance profile (individual antibiotic wise) from different substrates.

	Antibiotics	Sum of Squares	df	Mean Square	F
Nalidixic acid	Between Groups	6.000	2	3.000	0.081
	Within Groups	222.000	6	37.000	
	Total	228.000	8		
Cephalothin	Between Groups	0.889	2	444.	4.000
	Within Groups	667.	6	111.	
	Total	1.556	8		
Tobramycine	Between Groups	0.222	2	111.	0.009
	Within Groups	70.667	6	11.778	
	Total	70.889	8		
Nitrofurantoinine	Between Groups	0.000	2	000.	0.000
	Within Groups	10.000	6	1.667	
	Total	10.000	8		
Piperacillin	Between Groups	6.000	2	3.000	0.500
	Within Groups	36.000	6	6.000	
	Total	42.000	8		
Cefotaxime	Between Groups	978.667	2	489.333	74.644*
	Within Groups	39.333	6	6.556	
	Total	1018.000	8		
Amikacine	Between Groups	14.000	2	7.000	1.500
	Within Groups	28.000	6	4.667	
	Total	42.000	8		
Amoxycillin	Between Groups	200.000	2	100.000	17.647*
	Within Groups	34.000	6	5.667	
	Total	234.000	8		
Ciprofloxacin	Between Groups	288.667	2	144.333	11.809*
	Within Groups	73.333	6	12.222	
	Total	362.000	8		
Chloramphenicol	Between Groups	118.222	2	59.111	9.673**
	Within Groups	36.667	6	6.111	
	Total	154.889	8		
Cefozolin	Between Groups	16.889	2	8.444	1.617
	Within Groups	31.333	6	5.222	
	Total	48.222	8		
Pefloracin	Between Groups	64.889	2	32.444	20.857*
	Within Groups	9.333	6	1.556	
	Total	74.222	8		

\* Significant at 0.01 level, \*\* Significant at 0.05 level

be due to the comparatively higher nutritional status, availability of substrate for attachment or the positive interact of organism present in the sediment. It was suggested that association of vibrio with sediment was to overcome the unfavourable environmental

conditions (21).

*Vibrio parahaemolyticus* is the leading cause of seafood-associated bacterial gastroenteritis and is a moderately halophilic, salt-requiring bacterium. Global gene expression profiles of *V. parahaemolyticus*

**Table 2.** DMRT results for antibiotic resistance in *V. parahaemolyticus*.

Substrate	Water	Sediment	Shrimp
Nalidixic acid	20.00 <sup>a</sup>	22.00 <sup>a</sup>	21.00 <sup>a</sup>
Cephalothin	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.67 <sup>a</sup>
Tobramycine	20.67 <sup>a</sup>	21.00 <sup>a</sup>	21.00 <sup>a</sup>
Nitrofurantoinine	8.00 <sup>a</sup>	8.00 <sup>a</sup>	8.00 <sup>a</sup>
Piperacillin	26.00 <sup>a</sup>	27.00 <sup>a</sup>	28.00 <sup>a</sup>
Cefotaxime	13.33 <sup>a</sup>	36.67 <sup>b</sup>	34.00 <sup>b</sup>
Amikacine	20.00 <sup>a</sup>	23.00 <sup>a</sup>	22.00 <sup>a</sup>
Amoxicillin	10.00 <sup>a</sup>	20.00 <sup>b</sup>	20.00 <sup>b</sup>
Ciprofloxacin	30.67 <sup>b</sup>	30.00 <sup>b</sup>	18.33 <sup>a</sup>
Chloramphenicol	19.33 <sup>b</sup>	20.00 <sup>b</sup>	12.00 <sup>a</sup>
Cefozolin	12.00 <sup>a</sup>	13.33 <sup>a</sup>	15.33 <sup>a</sup>
Pefloracin	25.00 <sup>b</sup>	19.67 <sup>a</sup>	25.67 <sup>b</sup>

<sup>a</sup>: Means with similar letter as superscript are homogeneous.

grown under 2% and 0.66% NaCl were compared to define the low-salt stimulus (22).

In the present study the *V. parahaemolyticus* from sediment were more susceptible to the antibiotics as compared to the water and shrimp samples. A noteworthy observation of the present study is the incidences of large number of antibiotic resistant strains of *V. parahaemolyticus* from water. Most of the antibiotics administered in the present study were based on earlier reports. However, in total contrast *V. parahaemolyticus* isolated from water had the capacity to resist the antibiotic action. This indicates that the strains available in waters of Ponnani are more drug resistant than those reported from other parts of Kerala (13). Although in the present study no attempts were made to ascertain the pathogenicity of the isolates based on virulent genes, it would be

worth investigating whether virulence genes (*tdh* and *tlh*) are responsible for pathogenicity as described in earlier studies (17). In India, shrimp sample have been reported to harbor heavy load of vibrios (23). Although the incidence of *V. parahaemolyticus* from shrimp samples of Ponnani may not be in an alarming situation, it is worth mentioning the high numbers of multiple antibiotic resistant strains that were encountered in the present study.

Studies of *Vibrio* as a flora of seawater are mainly reported from temperate regions (9, 14). Tropical oceans and its inhabitants were reported to be good reservoirs of *Vibrio* species. Distribution of *Vibrio* species is influenced by the changes in the physico-chemical and ecological parameters. Quantitatively coastal water harbors more *Vibrio* than the open sea. However, recent reports points out that this

**Table 3.** Antibiotic resistance pattern in *V. parahaemolyticus* from different substrates.

	Substrate	Total isolate	Susceptible	Cluster 1	Cluster 2	Cluster 3	Multiple antibiotic resistant isolate	Antibiotic Resistance index
1	Water	12	5	3	4	0	7	0.032
2	Sediment	12	8	4	0	0	4	0.021
3	Shrimp	12	6	3	3	0	6	0.023



species may as well be found in low saline waters. *V. parahaemolyticus* were isolated from river mouths and coastal environments of average salinity of  $4.4 \pm 2.0$  ppt (24). Higher occurrence of *V. parahaemolyticus* were also observed during periods of lower salinity in autumn, with a total of 61 positive samples (18%) and a mean density of 1,234 most probable number/100 g. *V. parahaemolyticus* was primarily detected in areas of reduced salinity close to freshwater discharge points, where it was found in up to 45% of the samples (25).

The microbial status of seafood after catch is closely related to environmental conditions and the microbiological quality of the water. These factors include water temperature, salt content, distance between localization of catch and polluted areas, especially those containing human and animal feces, natural occurrence of bacteria in the water, ingestion of food by fish, methods of catch and chilling, and postharvest handling or processing conditions (18). Fish and seafood also may be a vehicle for many bacterial pathogens (10). In assessing the risks from fish, it is important to have information on the incidence of these pathogens. Temperature acts as a crucial factor affecting the occurrence of pathogenic forms of *V. parahaemolyticus* as observed from eight pelagic and five demersal fishes being sold at various outlets of Cochin (10). The present study was also conducted during summer season as a result the increased occurrence of pathogenic forms of *V. parahaemolyticus* from Ponnani may be due to increased temperature of coastal waters.

Results of antibiotic resistance index are comparable with those observed for *Vibrios* from coastal and brackish water areas of Kerala (16). While identifying the most effective antibiotic for *V. parahaemolyticus* it was seen that of the 12 antibiotic studied two i.e., cephalothin and nitrofurantoinine, recorded 100% drug resistance. However, tobramycin, piperacillin and amikacin were effective in curbing the growth of *V. parahaemolyticus*. Earlier studies from nearby areas resistant strains against ampicillin, tetracyclin, trimethoprim, nalidixic acid, ciprofloxacin and furazolidone (13). This indicates that coastal waters of Ponnani are infested with drug resistant groups of vibrios. Hence with what little information is available on the drug resistance of *V. parahaemolyticus* from Ponnani water, it is highly imperative to study the multiple drug resistance of this group for ensuring future seafood quality from the region and also to identify effective new generation antibiotics to curb

its pathogenicity. The work is particularly pertinent since most of the *Vibrio* strains isolated from the region have shown drug resistance to first generation antibiotics such as ampicillin. Since a benchmark study to this respect is lacking from Ponnani, it would be worth observing how the vibrios in water in general and shrimps in particular would evolve especially with the advent of resistant bacteria groups. It is found that there is a risk of transfer of resistant bacteria to human from consumption of aquaculture products and antimicrobial resistance is present in isolates from aquaculture environment (26). Isolated strains of *V. parahaemolyticus* from sediment and water sample have shown to be least resistance to chloramphenicol (19). Under the above perspective the importance of the study still grows since with changing environment more dominant groups establish and if Ponnani needs to transform itself as a seafood hub of Kerala, it has to curb the outburst of pathogenic *Vibrios* to ensure a safe export of seafood.

## CONCLUSION

The incidence of *V. parahaemolyticus* infection has been increasing in many parts of the world, due to the emergence of O3:K6 serotype carrying the *tdh* gene which is responsible for most outbreaks worldwide. Ponnani being one of the leading fish landing centers of India has the opportunity to transform as a major seafood hub, however, while assessing the quality of coastal water it is usually below par to the international standards prescribed for seafood processing. Present study clearly shows that the sediment in the region has been acting as reservoirs for *V. parahaemolyticus* which later gets transmitted to the overlying water and finally the shrimps that reach here for processing. Except for cephalothin and nitrofurantoinine no other antibiotics were found effective against most of these strains. Study also shows that a significantly larger number of resistant isolates for tobramycin, piperacillin and amikacin in the region. The multidrug resistant nature of *V. parahaemolyticus* strains adds to the gravity of the situation. Since fish and shrimps from Ponnani harbor are the main source of seafood for most of the population in this coastal region any incidence of emergence of drug resistant strains need to be addressed with caution. The present study provides a bird's eye view of the microbial population from three substrates in Ponnani and the data is envisaged to be a benchmark

for developing strategies for ensuring seafood safety through adoption of better management practices for ensuring high quality seafood for both domestic and international markets.

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