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Prevalence and distribution of *Vibrio parahaemolyticus* in finfish from Cochin (south India)

Santha Sudha[1], Puthenkandathil S. Divya[1], Bini Francis[1] & Ammanamveetil A.M. Hatha[1, 2]

Summary

Finfish samples obtained from four retail outlets in Cochin between June 2009 and June 2010 were investigated for the occurrence of *Vibrio parahaemolyticus*. A total of 182 samples were collected and suspect isolates were identified using standard biochemical tests and were further confirmed by a species-specific *tlh* gene. *V. parahaemolyticus* was detected in 45.1% of samples, with demersal fish being more affected than pelagic species. The bacterium was isolated more frequently from the skin and gills of pelagic fish, while the intestine yielded greater numbers of *V. parahaemolyticus* in demersal fish. The highest incidence of antibiotic resistance was recorded against ampicillin and streptomycin, followed by carbenicillin, cefpodoxime, cephalothin, colistin and amoxycillin; the lowest was against nalidixic acid, tetracycline, chloramphenicol and ciprofloxacin. Multiple drug resistance was prevalent among isolates. Although only a fraction of strains are pathogenic for humans, the time-temperature abuse in markets provides ample scope for these strains to multiply to dangerous levels. The multidrug resistant nature of the strains adds to the gravity of the problem. High *V. parahaemolyticus* incidence rates in market finfish samples from areas in and around Cochin clearly indicates that control measures should be adopted to reduce post-harvest contamination in seafood and time-temperature abuse in markets to diminish the risk of *V. parahaemolyticus* infection associated with seafood destined for human consumption.

Keywords

Antibiotic resistance, Cochin, Demersal fish, India, Pelagic fish, Retail market, *Vibrio parahaemolyticus*.

Prevalenza e distribuzione di *Vibrio parahaemolyticus* in pesci, Cochin (India del sud)

Riassunto

Campioni di pesci prelevati da quattro punti vendita a Cochin tra giugno 2009 e giugno 2010 sono stati esaminati per la presenza di Vibrio parahaemolyticus. *Un totale di 182 campioni sono stati raccolti e gli isolati sospetti sono stati identificati utilizzando test biochimici standard ulteriormente confermati con il gene specie-specifico* tlh. V. parahaemolyticus *è stata rilevato nel 45,1% dei campioni e i pesci demersali sono risultati più colpiti rispetto alle specie pelagiche. Il batterio è stato isolato più frequentemente da pelle e branchie dei pesci pelagici, mentre nei demersali il* V. parahaemolyticus *è stato isolato principalmente nell'intestino. La più alta incidenza di resistenza agli antibiotici è stato registrata nei confronti di ampicillina e streptomicina, seguite da carbenicillina, cefpodoxima, cefalotina, colistina e amoxicillina; la più bassa nei confronti di acido nalidixico, tetraciclina, cloramfenicolo e ciprofloxacina. La resistenza ai farmaci multipli è risultata prevalente tra gli isolati. Sebbene solo una*

(1) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Cochin 682 016, Kerala, India
mohamedhatha@gmail.com
(2) Fulbright Scholar: Environment Genomics Laboratory, Civil and Environmental Engineering, Michigan State University, East Lansing, MI 48824, United States (to 31 December 2012)

frazione di ceppi siano patogeni per l'uomo, il tempo-temperatura nei mercati lascia un ampio margine a questi ceppi per una moltiplicazione pericolosa. La natura farmacoresistente dei ceppi accresce la gravità del problema. Alti tassi di incidenza di V. parahaemolyticus *in campioni provenienti dal mercato ittico di Cochin e dintorni indica chiaramente che devono essere adottate misure di controllo per ridurre la contaminazione post-raccolta di pesci e di tempo-temperatura nei mercati per ridurre il rischio di infezione da* V. parahaemolyticus *associato ai pesci destinati al consumo umano.*

Parole chiave

Antibioticoresistenza, Cochin, India, Mercato al dettaglio, Pesce demersali, Pesce pelagico, *Vibrio parahaemolyticus*.

Introduction

Seafood constitutes one of the fastest growing sources of food. Billions of people throughout the world rely on fish as a primary source of protein, particularly in developing countries. Seafood is also the most important food commodity exported from developing countries. In the last two decades, there has been an increased awareness of the nutritional and health benefits of fish consumption. With increased fish consumption, there is also an increase in the number of foodborne illnesses. Seafood is known to be responsible for a significant percentage of foodborne diseases worldwide. Studies conducted in various countries, such as Malaysia (24), Brazil (42) and the United States (43), have indicated the occurrence and contamination of fish by various types of bacteria of which the most common pathogens isolated were *Vibrio* species. *Vibrio* species are natural inhabitants of aquatic environments and seafood harvested from contaminated waters, or which have been improperly preserved after harvesting, are known to play an important role in infections by members of this species (4). However, not all *Vibrio* species are pathogenic. Of the 65 species of the genus *Vibrio*, 12 are recognised as human pathogens (50), with 8 species considered to be directly associated with food (55). Of these, three species (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) are the most important and are responsible for most cases of foodborne illness (3).

Vibrios of seafood origin have attracted increasing attention as they are an important cause of food poisoning in humans (58). Among pathogenic vibrios that cause foodborne illnesses, *V. parahaemolyticus* causes the highest number of seafood-associated gastroenteritis in the United States and Asian countries (47), apart from few cases mediated by *V. alginolyticus* (16).

This is a Gram-negative, halophilic bacterium that is widely distributed in temperate and tropical coastal waters throughout the world in all varieties of finfish and shellfish (19) and is recognised as an important seafood-borne pathogen throughout the globe (59). Since 1996, cases of *V. parahaemolyticus* have increased worldwide, with the emergence of a highly virulent pandemic O3:K6 clone which is responsible for 50% to 80% of the recent *V. parahaemolyticus* outbreaks, including epidemics in India, France, Russia, South-East Asia, Japan and North America (46, 51, 53, 65). It has now become one of the most prevalent foodborne pathogens in countries where seafood is consumed in a variety of ways, including raw (8, 36, 40). Of the seafood-borne gastroenteritis cases in Japan, 70% are attributed to *V. parahaemolyticus* (20) and, in India, this organism accounts for about 3.5%-23.9% of gastroenteritis cases admitted to the Infectious Diseases Hospital in Kolkata (57).

However, not all of the environmental strains are considered to be pathogenic. The pathogenicity of *V. parahaemolyticus* in humans is associated with the production of thermo-stable direct hemolysin (TDH) and TDH-related hemolysin (TRH) encoded by *tdh* and *trh* genes, respectively (34). The frequency of tdh or trh detection among *V. parahaemolyticus* isolates from environmental samples and seafood was reported to be very low (2%-3%) in comparison to clinical isolates (99%) (52, 55). In recent years, some investigators have reported a higher prevalence of 10.2% and 12.8% (19, 22).

Consumption of raw or undercooked seafood contaminated with 10^7-10^8 organisms of *V. parahaemolyticus* may lead to the development of acute gastroenteritis characterised by diarrhoea, headaches, vomiting, nausea, abdominal cramps and low fever (12, 35, 66). In addition, *V. parahaemolyticus* also can cause wound infection in people exposed to contaminated seawater (49). In spite of the 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 the presence of sufficient bacteria in foods to cause disease. Although there is uncertainty concerning infectious doses (26), it is recognised that the general population is susceptible to infection by this organism (26). Antimicrobial therapy has been shown to reduce the duration and severity of symptoms of *Vibrio* infections in severe cases (34).

Emergence of drug-resistant bacteria is a global concern that threatens public health because of widespread use of antibiotics and resultant selection pressure for the emergence of resistant strains (18). Traditionally, *Vibrio* was considered highly susceptible to virtually all antimicrobials (54). The prevalence of antimicrobial resistance among foodborne pathogens has increased during recent decades and, in *Vibrio* spp., it has been observed to be dynamic and varies with the environment (56).

Cochin is regarded as the capital of India's seafood industry and accounts for over 90% of statewide exports. Fish forms a staple part of the human diet in this region. Although the occurrence of potentially pathogenic vibrios in market seafood has been documented in foreign countries, studies on the prevalence of this organism in finfish are meagre (21, 23). Retail surveys related to prevalence of *V. parahaemolyticus* in finfish are limited and offer little confirmation to the consumer regarding market seafood safety.

Considering the unhygienic market conditions, as well as the high degree of time-temperature abuse, the present study was conducted with the objective of determining the cross-contamination of finfish samples with *V. parahaemolyticus* sold at various outlets (roadside fish stalls, local markets and fish shops) in and around Cochin City. Prevalence in different regions of the body, such as body surface, gills and alimentary canal of pelagic and demersal fish, were analysed and the confirmed *V. parahaemolyticus* isolates were subjected to antibiotic sensitivity testing to ascertain the risk posed by these organisms. This information will be helpful to local consumers regarding the safety of various fish species consumed in this region and will ultimately help the seafood industry to strive for greater food safety.

Materials and methods

Sample collection processing and enrichment

Common outlets for procuring fish

Local markets (outlet 1, Ernakulam): these fish markets are easily accessible and are located around the periphery of the city limits. Fish sold at these outlets are usually kept on ice and are washed with water during display and sale.

Roadside fish stalls (outlet 2, Fort Cochin and outlet 3, Thoppumpady): here the fish vendors sell fish along the roadsides to enable easy accessibility for consumers. They display fish on the roadside by laying bundles of fish on tarpaulin sheets with no proper chilling conditions.

Fish shops (outlet 4, Tripunithura): fish shops are considered to be the best source for purchasing fish for domestic consumption. This is because the fresh fish is sold in a closed shop where there is exposure to dust and environmental contaminants. In these shops, fish are displayed on steel racks with proper chilling equipment. The sampling locations are shown in Figure 1.

A total of 182 finfish samples were collected from four retail fish outlets situated in and around Cochin, south India, at monthly intervals from June 2009 to June 2010. Finfish samples included *Caranx melampygus*, *Cynoglossus* spp., *Escualosa thoracata*, *Lactarius lactarius*, *Leiognathus* spp., *Nemipterus japonicus*, *Otolithoides biauritus*, *Pampus argenteus*, *Sardinella longiceps*, *Rastrelliger kanagurta*, *Parastromateus niger*, *Stolephorus indicus* and

Thryssa malabarica. Individual fish were collected in sterile polyethylene bags and brought to the laboratory in an ice box. Direct contact of the fish samples with the ice was avoided to ensure maximal survival and recovery of *V. parahaemolyticus*. All samples were processed within 2 h of collection. Aseptic procedures were strictly followed during collection, transportation and analysis of the fish samples. Isolation of *V. parahaemolyticus* was performed using the conventional isolation method (27). Briefly, swab samples of skin surface and gill from each finfish were enriched in 10 ml of alkaline peptone water (APW) with 3% NaCl. A 25 g sample of the gut of the was also homogenised with 225 ml APW with 3% NaCl in a sterile polythene stomacher bag (Masticator, IUL instruments, Barcelona) for 1 min, enriched at 37°C for 18 h-24 h.

Isolation and identification of *Vibrio parahaemolyticus*

After 18 h-24 h of incubation in APW broth, a loopful of each enrichment broth was aseptically streaked onto sterile surface dried thiosulphate citrate bile salt sucrose (TCBS) agar plates). The plates were incubated at 37°C for 24 h. Approximately 3-4 sucrose non-fermenting colonies that had a green or bluish green colour with dark blue or green centre measuring about 3-5 mm suggestive of *V. parahaemolyticus* were selected from the TCBS plates. These isolates were purified by restreaking onto TCBS agar and were then tested for sucrose fermentation in sterile sucrose broth supplemented with NaCl (3% w/v). Sucrose non-fermenting isolates were streaked onto sterile tryptone soy agar (TSA) slants supplemented with NaCl (3% w/v) and maintained at room temperature for further identification.

Halophilism tests were performed using tryptone broth with different concentrations of NaCl (0%, 3%, 6%, 8% and 10% w/v). Additional characterisation tests for the identification of *V. parahaemolyticus* namely: Gram staining, catalase, cytochrome oxidase, reactions on triple sugar iron and lysine iron agar, ability to produce arginine dehydrolase, lysine and ornithine decarboxylase were performed. Tests for glucose oxidation fermentation were performed using Hugh-Leifson broth and arabinose, lactose, mannitol, mannose, salicin, cellobiose and inositol fermentation tests were also performed following standard procedures (27). All media were supplemented with NaCl (3% w/v) unless otherwise specified. The presumptive positive cultures were further confirmed using HiCrome Vibrio Agar (HiMedia, Mumbai).

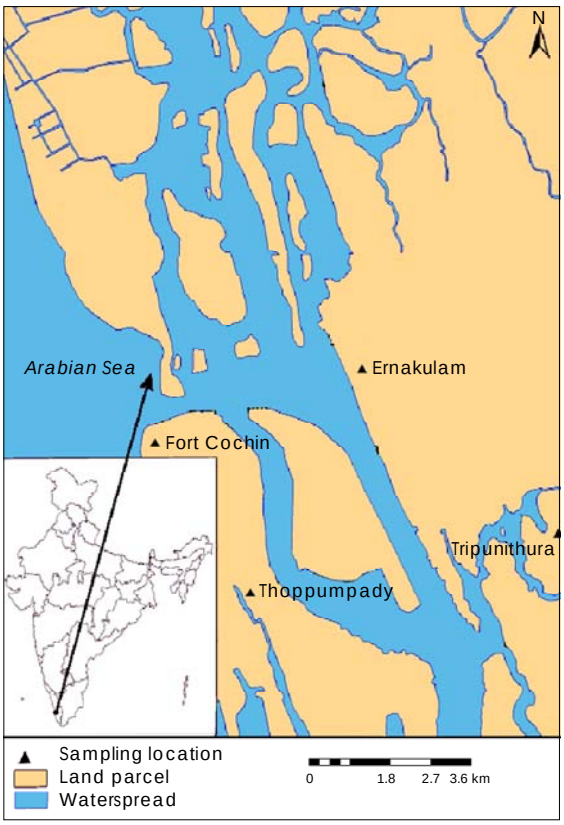

Figure 1
Location of fish markets in Cochin

Preparation of bacterial lysate and polymerase chain reaction assay

The polymerase chain reaction (PCR) for the species-specific *tlh* gene (450 bp) of *V. parahaemolyticus* was performed for confirmatory identification of the randomly selected isolates, essentially as previously described (7). *V. parahaemolyticus* strains obtained from the National Institute of Cholera and Enteric Diseases (NICED) in Kolkata was used as a known positive control for the PCR reaction.

Bacterial lysate was prepared following an established procedure described by Sujeewa *et al.* (62). Colonies of bacterial isolates from TSA+3% NaCl were mixed with 500 µl of sterile deionised water in Eppendorf tubes. This was mixed well by using a vortex mixer. The suspension was heated for 10 min in a water bath and then cooled on ice immediately. Cell debris from these cell lysates were pelleted by centrifugation (at 13 000 rpm for 2 min) and the supernatants were used as DNA templates in this PCR assay.

The PCR targeting species-specific *tlh* gene (450 bp) of *V. parahaemolyticus* was performed (7) in a thermocycler (Eppendorf Mastercycler, Hamburg) using a primer pair (5′ AAA GCG GAT TAT GCA GAA GCA CTG 3′ and 5′ GCT ACT TTC TAG CAT TTT CTC TGC 3′) to detect the gene fragment. PCR amplification was optimised in a total reaction volume of 25 µl consisting of sterile Milli Q water (13.5 µl), 10× PCR buffer (2.5 µl), forward primer (1.5 µl), reverse primer (1.5 µl), dNTP mix (0.5 µl, 200 mM), template (5 µl), and *Taq* DNA polymerase (0.5 µl). Components were mixed well and the PCR amplification of the target sequence was loaded into a thermocycler (Eppendorf Mastercycler, Hamburg), and programmed for 30 cycles of amplification. Each cycle consisted of three step reactions, initial denaturation (94°C, 3 min) followed by 30 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min) and extension (72°C, 1 min) followed by final extension (72°C, 5 min). The PCR products were resolved using agarose (1.5% w/v) gel electrophoresis. The gel was stained with ethidium bromide (0.5 mg/ml) and visualised under a UV transilluminator (AlphaImager, Innotech Corporation, San Leandro, California). A 100 bp DNA ladder was used as a molecular size marker.

Antibiotic sensitivity test

Antibiotic sensitivity of the *V. parahaemolyticus* cultures was determined by the standard disc diffusion method (6). The 14 antibiotics tested for this assay were those for which breakpoints for *Vibrio* spp. have been established (15). Antibiotic discs (HiMedia, Mumbai) were placed aseptically on the Mueller-Hinton agar plates pre-inoculated with the test culture. The discs were placed at equal distance, and sufficiently separated from each other to avoid overlapping of the zone of inhibition. The antibiotics evaluated were ampicillin (10 µg), amoxycillin (30 µg), carbenicillin (100 µg), cefpodoxime (10 µg), ceftazidime (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), colistin (10 µg), gentamicin (10 µg), nalidixic acid (30 µg) streptomycin (30 µg), tetracycline (30 µg) and trimethoprim (5 µg). The plates were incubated at 37°C for 18 h-24 h, and the size of inhibition zone was recorded. Controls, consisting of media without the antimicrobial discs and inoculated with the test micro-organisms, were used in all experiments. An *E. coli* strain ATCC 25922 with known sensitivity patterns was included as a positive control. Inhibition zones were interpreted using Clinical and Laboratory Standards Institute recommendations (14).

Results

The incidence of *V. parahaemolyticus* in finfish was found to be 45.1% by conventional biochemical tests. A total of 82 isolates which were confirmed to be *V. parahaemolyticus* by HiCrome vibrio agar, were further confirmed by PCR targeting the *tlh* gene. The isolates were found to amplify *tlh* gene, thus confirming the identity of the bacteria (Fig. 2).

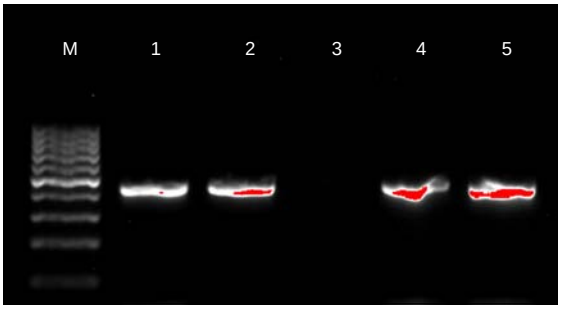

Figure 2
Polymerase chain reaction for the detection of *tlh* gene of *Vibrio parahaemolyticus* (450 bp)
Lane M: DNA ladder (100 bp)
Lane 1: positive control
Lanes 2, 4, 5: *Vibrio parahaemolyticus* isolates from finfish samples containing *tlh* gene

There was individual species variation among finfish in the occurrence of *V. parahaemolyticus* (Table I). The prevalence level was found to vary from 14.2% in *T. malabarica* to 75% in *P. argenteus.* The bacteria were isolated from *C. melampygus Cynoglossus* spp., *E. thoracata, L. lactarius, Leiognathus* spp., *N. japonicus, O. biauritus, P. argenteus, S. longiceps, R. kanagurta, Parastromateus niger, S. indicus* and *T. malabarica.* Among these, consumption, as well as contamination frequencies of *P. argenteus, N. japonicus, R. kanagurta* and *S. longiceps* were high, posing a potential health risk to the consumers. Consumption of other fish examined is low, partly because of limited availability in the local outlets. Contamination of *T. malabarica, S. indicus* and *L. lactarius* samples with this bacterium were comparatively low.

The occurrence of *V. parahaemolyticus* was varied significantly at different regions of the finfish samples analysed. It was most prevalent in the gut region (48.8%), followed by gill tissue (30.5%) and skin (20.7%). There were also variations in the distribution of *V. parahaemolyticus* on the body parts of pelagic and demersal fish species. *V. parahaemolyticus* was more frequently encountered on the skin and gill of pelagic fish, while intestinal samples yielded more positive isolations in demersal fish (Table II).

Table II
Frequency of recovery from fish that were positive for *Vibrio parahaemolyticus* from different retail outlets

Collection centre	Percentage recovery
Outlet 1	13.7
Outlet 2	78.6
Outlet 3	75.5
Outlet 4	12.5

When the contamination frequencies of the fish collected from the four retail outlets were compared, it was found that the fish collected from outlets 2 and 3 showed greater recovery levels of *V. parahaemolyticus* than those recovered from outlets 1 and 4 (Table III).

Table III
Prevalence of *Vibrio parahaemolyticus* in the body parts of demersal and pelagic fish

Body parts	Prevalence
Demersal fish	
Skin	11.8%
Gill	8%
Gut	92.5%
Pelagic fish	
Skin	88.2%
Gill	92.0%
Gut	7.5%

Table I
Occurrence of *Vibrio paraahemolyticus* in different finfish

Name of fish	No. of samples examined	No. of samples contaminated (%)
Demersal fish		
Pampus argenteus	8	6 (75.0)
Parastromateus niger	6	4 (66.7)
Nemipterus japonicus	17	11 (64.7)
Cynoglossus spp.	15	9 (60.0)
Leiognathus spp.	19	11 (57.9)
Pelagic fish		
Rastrelliger kanagurta	26	15 (57.7)
Otolithoides biauritus	8	4 (50.0)
Sardinella longiceps	24	10 (45.8)
Caranx melampygus	10	3 (30.0)
Escualosa thoracata	18	4 (22.2)
Lactarius lactarius	11	2 (18.2)
Stolephorus indicus	13	2 (15.4)
Thryssa malabarica	7	1 (14.3)

When tested for their susceptibility to various antibiotics, most of the isolates were resistant to ampicillin (89%) and streptomycin (89%), followed by carbenicillin (83%), cefpodoxime (80%), cephalothin (80%), colistin (77%) and amoxycillin (63%). All the isolates tested remained susceptible to nalidixic acid (100%) and tetracycline (100%). More than 70% of the isolates were sensitive to chloramphenicol, trimethoprim, ceftazidime and gentamicin, respectively (Table IV).

Discussion

Seafood is known to be responsible for a large number of foodborne diseases worldwide (37). *Vibrio* species account for a significant proportion of human infections from the consumption of raw or undercooked seafood (39). Among the pathogenic *Vibrio* species, *V. parahaemolyticus* is an important seafood-borne pathogen and, in India, the incidence is reported to have doubled in the past five years (13). In our study, a series of biochemical tests and a molecular approach using *tlh* targeted PCR were used for the identification of *V. parahaemolyticus*. All the biochemically confirmed isolates were found to possess the species-specific *tlh* gene. Our study revealed that the *tlh* gene was a useful target for the confirmation of *V. parahaemolyticus* from the study region in India. This concurs with the earlier reports of *tlh* gene as a species-specific marker for *V. parahaemolyticus* (7, 44, 63, 64).

The skin surface, gills and intestine are the established niches for the colonisation of *Vibrio* (9). The major components of skin flora were similar to those in the ambient water, indicating a reflection of their environment. Gills being site of exchange for large quantities of water tends to concentrate large numbers of bacteria. When these microbes are provided with favourable conditions for growth, they establish on the gills. Handling methods and pre-capture environment also affect surface flora (32).

The subsurface of live fish is bacteriologically sterile, as the immune system prevents bacteria from growing in the flesh. Bacteria gain entry only when the fish dies and then they proliferate freely (33). Fish flesh is an excellent substrate for growth of a wide range of micro-organisms. The bacteria which are able to establish themselves on the skin and the gills have the ability to gain entry again into the inner parts of the fish, such as the gut. All fish can carry certain levels of bacteria and the incidence of these microbes in fish are vastly affected by the geographic location, time of holding and temperature abuse during handling (33).

Table IV
Percentage of resistance of *Vibrio parahaemolyticus* to different antibiotics

Antibiotic	Percentage of *Viibrio parahaemolyticus* strains that are		
	Resistant	Intermediate	Sensitive
Ampicillin	89	11	0
Amoxicillin	63	14	23
Carbenicillin	83	8.5	8.5
Cefpodoxime	80	20	0
Ceftazidime	0	26	74
Cephalothin	80	11	9
Chloramphenicol	0	3	97
Ciprofloxacin	0	54	46
Colistin	77	0	23
Gentamicin	0	29	71
Nalidixic acid	0	0	100
Streptomycin	89	11	0
Tetracycline	0	0	100
Trimethoprim	0	11	89

The occurrence rate of *V. parahaemolyticus* observed in finfish in the present study is 45.1%. A similar incidence rate in 42% of the finfish samples from the same area has also been reported (10). However, the recovery rate is higher than those reported in the previous works from the south-west coast of India (19, 23, 60, 61). *V. parahaemolyticus* occurrence varied according to finfish species. The bacteria was more prevalent in *P. argenteus*, *P. niger*, *N. japonicus* and *Cynoglossus* spp.; this was due to their resident behaviour as all these fish are demersal in nature. When fish were grouped according to their residence behaviour, the bacterium was isolated from 63.1% of demersal species and 35.0% of pelagic species. However, indications on the definite risk associated with *V. parahaemolyticus* could be ascertained only after testing virulence-specific genes. Although a fraction of the total *V. parahaemolyticus* is actually pathogenic, the prevailing time-temperature abuse in the retail markets of the study area, coupled with the short generation time of this organism, offer ample scope for this fraction to multiply and pose a risk to the consumer. In accordance with the rules of the Export Inspection Council (EIC) of the Government of India, there is no tolerance for *V. cholerae* and *V. parahaemolyticus* in seafood destined for export, despite their ability or inability to produce virulence factors.

The occurrence of *V. parahaemolyticus* was significantly different in different sections of the fish samples analysed. It was more prevalent in the gut region of most of the demersal fish samples. *V. parahaemolyticus* could be isolated from the skin and gill of most of the pelagic fish, but not from the demersal fish. It was suggested that the high incidence (48.8%) in intestines resulted from the fact that the intestinal tract provides a micro-environment for the survival of the bacterium (61). Since bacterial flora of a fish is a reflection of the environment in which it is caught, this evidently indicates that difference in habitat of pelagic and demersal fish could explain the variations in the distribution of *V. parahaemolyticus.* Prevalence of *V. parahaemolyticus* in demersal fish has been reported and further investigations are needed to determine the factors affecting the differential presence of *V. parahaemolyticus* in demersal and pelagic fish species (17).

Studies performed have demonstrated that microbial contamination in fish was mainly caused by a lack of sanitary procedures in handling fish during transit from the sea to the market and also during the sale of fish (29). At retail markets, the fish skin and gills are in direct contact with microbial contaminants in the environment. This condition may accelerate the growth of most of the pathogenic bacteria. Fish displayed on ice chests during sale could further increase the risk of microbial contamination. The growth of pathogenic micro-organisms was favoured in warmer temperatures. This suggests that rapidly chilling fish post harvest could reduce possibility of proliferation of these organisms. Previous studies have also indicated that fish should be kept in cold conditions during transit and storage to reduce the risk and level of *Vibrio* presence (24).

V. parahaemolyticus contamination in fish was significantly different at different retail outlets. Fish sold at roadside stalls (Nos 2 and 3) were found to be highly contaminated. In these stalls, fish vendors were selling fish next to the roadside to provide easy access for the customers. Furthermore, it is a cheap mode of displaying and selling fish compared to the fish markets, where stall fees are required. Fish vendors display fish on the roadsides by laying the fish bundles on paper boards and pieces of tarpaulin pieces with no proper cold or chilling equipment or storage techniques. They are mostly exposed to ambient temperatures and with dust and pollution from motor vehicle emissions. Since the climatic conditions are almost always warm, time temperature abuse of the seafood might have resulted in the multiplication of *V. parahaemolyticus*. Temperature is a crucial factor in the multiplication of bacteria. Recent findings have also shown that, at ambient temperature, the microflora at the point of spoilage is dominated by mesophilic *Vibrionaceae*. In outlets 1 and 4, fish were usually washed with water and kept on ice during display and sale, which is not practised by the fish sellers near the roadside stalls.

Previous investigators have also reported that the levels of *V. parahaemolyticus* increases in seafood that are exposed to temperatures ranging from 20°C to 30°C. If the seafood is exposed to temperatures at 20°C to 35°C, moderate levels of bacteria can increase to high levels within 2 h-3 h (1). Cochin usually lies within this temperature range and this may explain the presence of high levels of *V. parahaemolyticus* in the samples.

Storing fish in direct contact with ice is damaging to *V. parahaemolyticus* (38). This is confirmed in our study which shows that fish sold in outlets 1 and 4 had a lower level of contamination as fish were stored at low temperatures. Reports have indicated that *V. parahaemolyticus* cells are injured at temperatures of 0°C-5°C. This implies that efficient monitoring of temperatures in fish markets should be practised.

Researchers have investigated the presence of *V. parahaemolyticus* in retail pre-packed portions of marine fish sold in Spain (30) and Japan (48) and showed that improper handling and processing of fish were major contributors to fish contamination by *V. parahaemolyticus*. In India, the physical facilities and infrastructure in all types of fish markets are far from satisfactory (25). Most fish landing centres and fish markets are old, crowded and have an excess number of traders selling fish, even in the passages and without proper infrastructure facilities, thereby resulting in poor fish handling. Most retailers were found selling fish by the roadside without observing either quality or hygiene. The high levels of *V. parahaemolyticus* recovered from outlets 2 and 3 may be liked to these reasons.

The widespread use of antibiotics in food animal production systems has resulted in the emergence of antibiotic-resistant bacteria that can be transmitted to humans through the food chain. The result of our study also confirms the prevalence of multiple drug resistance among *V. parahaemolyticus* strains. It was observed that a high percentage of the isolates tested (89%) were resistant to ampicillin, confirming that this is the drug that is widely used in animal production systems and present in contaminated environments. In China, *Vibrio* spp. (including *V. parahaemolyticus*) isolated from *Sparus sarba* has shown resistance to ampicillin (41). These results indicate that that first generation antibiotics, including ampicillin, have been thoroughly abused in the environment. Ampicillin is a drug approved by the Centers for Disease Control and Prevention (CDC) to treat *V. parahaemolyticus* infections. The high percentage of *V. parahaemolyticus* observed with reduced susceptibility to ampicillin suggests that this agent is no longer an appropriate drug for the treatment of *V. parahaemolyticus* infections (5).

Comparable levels of resistance to antibiotics directed against the infections caused by the members of the genus *Vibrio* have also been reported earlier; large numbers of isolates have demonstrated high levels of resistances to ampicillin, amoxicillin, streptomycin and crabenicillin (5, 28). According to the data from World Health Organization Expert Consultations on critically important anti-microbials for human medicine, amoxicillin, streptomycin and carbenicillin are considered to be critically important in human medicine to treat serious Gram-negative infections. Consequently, the relatively high percentage (89%, 89% and 83%, respectively) of resistance might be due to the fact that anthropogenic factors, such as hospital effluents, might have influenced resistance to these antibiotics in *Vibrio* spp. (45).

Interestingly, antibiotic resistance was lower against chloramphenicol (0%), tetracycline (0%), nalidixic acid (0%), gentamicin (0%) and trimethoprim (0%), which are commonly used in aquaculture farms through feed during culture and hatchery production of seeds. This poorly developed resistance to life-saving drugs, such as tetracycline, nalidixic acid trimethoprim, followed by chloramphenicol, has also been reported (11).

The Aquaculture Authority of India has banned about 20 antibiotics and pharma-cologically active substances, including chloramphenicol and nalidixic acid, for use on aquaculture farms (2). Thus, the ban imposed on these antibiotics for use on aquaculture

farms might have resulted in the low resistance to these antibiotics.

Conclusions

In conclusion, the results of our study revealed a high rate of prevalence of *V. parahaemolyticus* in pelagic and demersal fish collected from different markets at Cochin, south India. Based on the observations herein, it must be emphasised that seafood products at the retail level may contain potentially dangerous bacteria. Although the exact source of contamination has not been determined, some conditions from capture to market, such as lack of hygiene, improper handling, cross-contamination and time-temperature abuse can favour survival of the bacterium.

The multi drug-resistant nature of *V. parahaemolyticus* strains adds to the gravity of the problem, in case of foodborne outbreaks mediated through this organism. Fish being a popular food item among the local population warrants constant monitoring for quality issues, especially those related to bacteriological quality. To contribute to a better characterisation of this organism, future studies involving the determination of pathogenicity markers, such as *tdh* and *trh*, to determine the relative proportion of pathogenic strains, are essential and we have initiated further work in this regard. It is expected that the data will be valued for improving the processing strategies and enlarging the vision on the concept of high quality and safe seafood in domestic as well as international trade.

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