

Original Research Article

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Microbiological Quality of Fish and Shellfish, with Special Reference to *Vibrio parahaemolyticus* in Domestic Markets of West Bengal, India

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ABSTRACT

We studied the microbiological quality and prevalence of *Vibrio parahaemolyticus* in finfish and shellfish sold in the domestic markets of West Bengal, India following enrichment in alkaline peptone water and further plating on to thiosulfate citrate bile salt sucrose agar. Total plate count of bacteria in shrimp flesh with the exoskeleton, and finfish muscle with skin was also estimated which ranged from $20 \pm 1.12 \times 10^5$ cfu/g to $73 \pm 1.12 \times 10^5$ cfu/g, and from $0.2 \pm 0.18 \times 10^5$ cfu/g to $1.2 \pm 0.43 \times 10^5$ cfu/g, respectively. Prevalence of *V. parahaemolyticus* varied in different fish and shellfish species, with highest prevalence in *Lutjanus* sp (27.77%) and lowest in *Setipinnaphasa* (10%); the bacterium was not detected in *Labeobata* and *Channa striata*. The contamination rate was higher in shellfish like *Penaeus monodon* (42.85%) and *P. indicus* (40%). Contamination was higher during summer than in winter: an average of 20.85 % of finfish and shellfish were contaminated during winter, compared to contamination of 31.45% of samples during summer months. The study showed that although overall microbial load was within permissible limits, fish and shellfish were contaminated with *V. parahaemolyticus* raising food safety concern to the domestic consumers.

Keywords

Vibrio parahaemolyticus,
Penaeus monodon,
Setipinnaphasa

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Practical application/ significance of the study

Fish and shellfish are known to transmit many of the established food borne microbial infections and intoxications to human. India has meager information available on the occurrence of food contamination by pathogenic bacteria especially in domestic markets. The present study has given an

indication of a hygienic sanitary quality of finfishes and shellfishes available in domestic markets of West Bengal.

Wide occurrence of *V. parahaemolyticus* in raw fish and shrimps may pose a serious health risk for large fish-eating population of Bengal. Data on the uneven distribution of this bacterium in various fish and shellfish will provide a guideline for microbial safety of

different fish and shellfishes as well as fish processing industry towards better food safety.

Introduction

The microbiological quality of finfish and shellfish is important for consumer health. Both the total bacterial load and presence of pathogens determine quality and safety of fish/fish products. Seafood often harbors infectious agents that are naturally present in the aquatic environment or may be introduced through human intervention. Raw or undercooked seafood have been implicated as major vehicles of *Vibrio parahaemolyticus* infection to humans (Venkitanarayanan and Doyle, 2001). *Vibrio parahaemolyticus*, ahalophilic gram-negative bacterium is widely distributed in temperate and tropical coastal waters throughout the world (DePaola *et al.*, 2000) and some of its strains can cause gastroenteritis in humans through ingestion of contaminated seafood (Matsumoto *et al.*, 2000 ; Zarei *et al.*, 2012 ; Yano *et al.*, 2014). Seafood mainly crustaceans & mollusks are generally considered as reservoirs of this pathogen (Cook *et al.*, 2000; DePaola *et al.*, 2000; Daniels *et al.*, 2001; Lozano-Leon *et al.*, 2003). In different environmental conditions, this bacterium could thrive and proliferate in oysters (Gooch *et al.*, 2002). *V. parahaemolyticus* infection has been implicated as one of the major causes of food-borne illness in the world (Joseph *et al.*, 1983; Wong *et al.*, 2000) and in Asia (Chiou *et al.*, 2000; Chowdhury *et al.*, 2013; Kubota *et al.*, 2008; Ma *et al.*, 2014). Majority of the food poisoning cases have been reported due to *V. parahaemolyticus* infection in Taiwan, Japan, and several Southeast Asian countries (Chiou *et al.*, 1991)

West Bengal is a major fish producing as well as fish eating state in India where large majority of people eat fish. The state requires approximately 1.672 million tonnes fish per

annum. During 2014-15, the state produced 1.617 million tonnes fish of which 1.438 million tonnes from Inland and 0.179 million tonnes from Marine sector. West Bengal also has the largest impounded brackish water area where traditional and improved traditional shrimp farming are practiced. The total shrimp production in West Bengal was 115916 ton during 2014-15 (Department of Fisheries, Govt. of West Bengal 2014-15).

Unlike in developed countries, Indians prefer deep fried or boiled curry preparations of fish & seafood which markedly reduce microbial load, including *V. parahaemolyticus* in cooked foods. Despite this, prevalence of *V. parahaemolyticus* in human diarrhea cases in India has also been noticed by several researchers. In India, the first outbreak of *Vibrio parahaemolyticus* induced diarrhea in human was reported from Vellore in South India (Lalitha *et al.*, 1983). West Bengal, Kolkata and neighboring Bangladesh are well known for cholera and other endemic diarrhoeal outbreaks. Pal *et al.*, 1985 have reported about 3.5-23.9% of acute human diarrhea cases owing to *Vibrio parahaemolyticus* infection in Kolkata. During 2001-2012 *V. parahaemolyticus* has been isolated from 1.308% of diarrheal patients admitted at Infectious Disease Hospital, Kolkata (Pazhani *et al.*, 2014). *V. parahaemolyticus* diarrhea incident rate was 0.63% reported and isolated by Kanungo *et al.*, (2012) from urban slum in Kolkata during 2007-2010.

West Bengal is one of the major inland fish producing states, producing approximately 21-23% of inland fish production of India. Export of marine and aquaculture product from West Bengal for the year 2014-15 was 85138 ton, valued 609.05 Million US\$. (Department of Fisheries, Govt. of West Bengal 2014-15). Fish and shellfish going for export are processed that reduces their microbial load.

However, bulk fish and shellfish produce go into domestic markets without any processing that make them vulnerable to microbial contamination and poor quality. However, microbial quality of fish and shellfish sold in domestic markets are not routinely monitored. The present study has examined the microbiological safety of finfish and shellfish in domestic markets and prevalence of *Vibrio parahaemolyticus* in said samples from different retail fish markets in and around Kolkata

Materials and Methods

Finfish and shellfish collection

Fresh but ice preserved finfish species like *Latescalcarifer*, *Mugil cephalus*, *Channa striata*, *Channa punctata*, *Labeobata*, *Gudusia chapra*, *Pampus argenteus*, *Lutjanus* sp., *Setipinna phasa* and shrimps viz. *Penaeus monodon*, *Penaeus indicus* were collected aseptically in individual polypacks from domestic retail markets in and around Kolkata, West Bengal, India and brought to the laboratory on ice.

Total plate count of bacteria

Shrimp muscles including exoskeleton and finfish flesh were cut into small pieces aseptically with sterile scissors and homogenized in sterile normal saline (0.85% NaCl) to 10% w/v suspension. The homogenized tissue samples were then serially diluted in normal saline and plated onto nutrient agar (DIFCO, USA) by pour plate technique for total aerobic plate count (TPC) of bacteria and incubated at 37°C for 24 hours in a bacteriological incubator.

Ice is widely used for fish preservation, but commercially produced ice may be a source of bacterial contamination to the produce. To examine microbiological quality, ice samples

were collected aseptically from ice sellers in fish markets in sterile poly packs and 10 ml of ice sample was inoculated in 90 ml normal saline (0.85% NaCl) to 10% w/v suspension, serially diluted in normal saline and plated on to nutrient agar (DIFCO, USA) by pour plate technique for total aerobic plate count (TPC) of bacteria.

Isolation of *V. parahaemolyticus*

The procedure described by Food and Drug Administration (FDA, 2004) was followed with minor modifications for estimation of *Vibrio parahaemolyticus* in fish and shellfish samples. Briefly, 25g sample was homogenized in 225 ml alkaline peptone water (APW) (DIFCO, USA) and an aliquot was taken for making serial dilution and spread plating on TCBS agar (DIFCO, USA) plates. The enrichment broth was incubated at 37°C for 6 h and subcultured on TCBS agar. The TCBS agar plates were incubated at 37°C for 24 h. Presumptive *Vibrio parahaemolyticus* colonies having bluish-green color on TCBS agar plates were picked and identified by biochemical tests, viz. Oxidase test, sensitivity to O/129, growth in NaCl (1%, 3%, 6%, 8% and 10%), acid production from sucrose, d-cellobiose, lactose, arabinose and d-mannose, gelatinase and production of ornithine and lysine decarboxylase and arginine dihydrolases per the protocol recommended in the FDA Bacteriological Analytical Manual (FDA, 2004).

Identification of *Vibrio parahaemolyticus* isolates

The gene encoding 16S rDNA was PCR amplified from the presumptive *Vibrio parahaemolyticus* using universal bacterial 27f (5'-GAG TTT GAT CCT GGC TCA G-3') and 1492r (5'-TAC GGT TAC CTT GTT ACG AC-3') primers. The PCR reaction was performed in 50 µl volume containing 25 µl of

Red TaqReadyMix (Sigma), 22 µl water, 0.2µ each of forward and reverse primers and template DNA. The template DNA was obtained by extracting genomic DNA using the Gen Elute Bacterial Genomic DNA Kit (Sigma- Aldrich) from a fresh colony grown on nutrient agar. The following cycle was used for PCR reaction: initial denaturation at 95°C for 1 minute, followed by 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes and the final extension at 72°C for 5 minutes. The PCR amplicons were analyzed on a 1% agarose gel with 0.5 X TBE as the running buffer. A 100 bp standard DNA ladder (Sigma) was included on each gel for base pair size comparison (Figure 1). PCR products were sequenced from a commercial house by Sanger sequencing method. Sequenced data were edited and aligned using Codon Code Software and identification of isolates were determined following sequence homology in NCBI GenBank and RDP databases. The phylogenic relationship among the strains B2, B3, B4, B6 and B11 was determined with the closest type strains using MEGA (version 5.2) software following the minimum evolution method.

Statistical analysis

The bacterial loads in finfish and shellfish are expressed as mean + S.D and compared in different species using SAS 9.2 software at $\alpha = 0.05$ significance. The figures and graphs were drawn using JMP 8.0.2 software.

Results and Discussion

The total bacterial loads (cfu/g) in the muscle with the skin of finfish and shellfish in different domestic markets in and around Kolkata are presented in Table 1. The total bacterial load of finfish varied from $0.2 \pm 0.18 \times 10^5$ cfu/g to $1.2 \pm 0.43 \times 10^5$ cfu/g. The bacterial load of shrimp samples and ice varied from $20 \pm 1.12 \times 10^5$ cfu/g to $73 \pm 1.12 \times$

10^5 cfu/g and $0.3 \pm 0.03 \times 10^5$ cfu/ml to $1.0 \pm 0.12 \times 10^5$ cfu/ml respectively. Bacterial load in fish, shellfish or ice samples collected from different markets did not differ significantly in their bacterial loads; however bacterial load in shellfish samples were significantly higher than that in fin fish and ice samples.

The presumptive *V. parahaemolyticus* isolates obtained from finfish and shellfish were identified by a battery of biochemical tests. However, a few isolates differ in one or two biochemical reactions recommended by FDA create confusion among researchers in the identification of the organism (Bharathi *et al.*, 1987; Karunasagar *et al.*, 1997). Hence, to confirm identification, 16S rDNA sequence was used for correct identification of the isolates. The 16SrDNA sequences of representative isolates were submitted to GenBank and accession numbers obtained (JQ265999, JQ266001, and JQ266003). Based on the nucleotide homology and phylogenetic analysis of the 16S rDNA gene sequences, identification of B2, B3, B4, B6 and B11 strains were confirmed as *Vibrio parahaemolyticus*. The strains B6 and B11 showed 100% similarity while isolate B2, B3, B4 showed 99% similarity with *Vibrio parahaemolyticus* (Figure 2).

There were individual species variation among finfish and shellfish samples in their prevalence of *Vibrio parahaemolyticus* (Table 2). The bacterium was most commonly isolated from marine fishes, viz. *Lutjanus* sp (27.77%), *Pampus argentius* (16.66 %), estuarine fishes, viz. *Lates calcarifer* (21.42%), *Mugil cephalus* (12.5%), and less so in freshwater fishes, viz. *Gadusia chapra* (12.5%), *Channa punctatus* (11.11%), *Setipinnaphasa* (10%). Overall the bacterium was detected in 51(25.5%) samples. The contamination rate was higher in shellfish like *Penaeus monodon* (42.85%) and *Penaeus indicus* (40%). The distribution of *V.*

parahaemolyticus in finfish and shell fishes of different months are presented in Figure 3. It was also observed that *V. parahaemolyticus* load was higher in warmer months viz. March, April than colder months December and January.

The present study was conducted to assess the microbiological quality of finfish and shellfish in and around Kolkata and their safety for domestic consumers. The total bacterial load in fish and shellfish samples collected from various retail markets of West Bengal, India showed insignificant variation ($P > 0.05$) among retail market sites. Possible reason for this was that the markets surveyed were not very far from one another; source of the fish available in the markets might have originated from adjacent fish farms, *bheries* (low saline wetlands) and estuaries and within the same geo-climatic region. However, bacterial load in finfish, shrimp, and ice samples varied significantly ($P < 0.05$) with the highest load in shellfish. The bacterial loads in fish samples of Bengal were within the range of 10^4 cfu/g. Thus, most of the finfish samples examined from different markets were of acceptable quality, considering the TPC limits set by the ICSMF (1986), USFDA, EU, and Export Inspection Council of India. The bacterial loads of shrimp were more or less same in different fish markets and were in the range of $2-7.3 \times 10^6$ CFU/g. As per ICSMF guidelines (1986) shrimp from different markets were also acceptable. An earlier study undertaken at farm level had reported APC of whole shrimp, shrimp surface and gut of the shrimp to be 8.40×10^5 , 4.60×10^5 / cm^2 and 1.26×10^6 / g (Nayyarahamed *et al.*, 1994). Moreover, the bacterial loads in ice samples were recorded in the range of 10^3 cfu/g which showed insignificant variation for fish and shrimp contamination. The TPC of finfish and shellfish were found to be in conformity with the result as described by Manna *et al.*, (2008). The bacterial loads in shrimps were

higher than in fish samples, possibly as species variation or variation in microbiological quality of shrimp farms. In this region, the low to medium saline intensive shrimp farms are often contaminated with city sewage water to varying extents.

The present study also examined the prevalence of *V. parahaemolyticus* in finfish and shellfish collected from domestic markets in and around Kolkata. This organism was given importance as it is frequently implicated in diarrhoeal cases in human since 1996 and abruptly gained a new global dimension for its pathogenicity since (1997) (Okuda *et al.*, 1997). *V. parahaemolyticus* in various species of finfish and shellfish were detected in 25.5% of samples. The contamination rate was higher in shellfish (*P. monodon* 42.85%, *P. indicus* 40%) as compared to finfish (15.99%). In Kolkata, the prevalence of *V. parahaemolyticus* was earlier reported from shellfish (45.83%) and finfish (16.73%) (Das *et al.*, 2009). Out of 90 fish samples examined about 60 showed positive with *V. parahaemolyticus* containing virulence *tdh* gene in Kolkata (Pal and Das 2010). The bacterium was most commonly isolated from marine fishes and less so in freshwater fishes. No *V. parahaemolyticus* was detected in *Labeobata* and *Channa striata* samples and might be considered safe for human consumption. *V. parahaemolyticus* could infect wide host range starting from marine animals, fish to fresh water fish. Fish irrespective of their origin (marine and fresh water) act as an ideal substrate for the proliferation of *V. parahaemolyticus*. Based on early ecological studies occurrences of *V. parahaemolyticus* have also recorded in freshwater plankton and in freshwater fishes. The occurrence of *V. parahaemolyticus* from market samples of freshwater fishes was might be due to cross-contamination and mishandling by fishmonger (Sarkar *et al.*, 1985).

Table.1 Bacterial load, [cfu/g or cfu/ml] in finfish, shellfish and ice samples

	Market					
	Barrackpore	Garia	Howrah	Kolkata	Kalyani	Ranaghat
Fish^a	0.8±0.34	1.2±0.43	0.7±0.64	0.7±0.55	0.6±0.28	0.2±0.18
Shrimp^b	54±2.01	56±2.91	73±1.12	61±1.56	27±1.34	20±1.12
Ice	0.7±0.07	0.9±0.11	1.0±0.12	0.8±0.10	0.3±0.04	0.3±0.03

^a Fish (*Labeo rohita*, *Labeo bata*, *Gudusia chapra* etc).

^b Shrimps (*Penaeus monodon*, *Penaeus indicus*).

Bacterial load (Mean±S.D.) expressed as CFU x 10⁵/g fish or shellfish or CFU x 10⁵/ml ice; n = 5 for each fish/shrimp species and ice samples from each market.

Table.2 Prevalence of *V. parahaemolyticus* in different finfish and shellfish species

Fish /shellfish	Habitat of the fish / shellfish species	Number of samples examined	Number of samples contaminated	Percentage of samples contaminated
<i>Penaeus monodon</i>	Marine & estuarine	35	15	42.85%
<i>Penaeus indicus</i>	Marine & estuarine	45	18	40%
<i>Lutjanus Sp. (Snapper)</i>	Marine & coastal water	18	5	27.77%
<i>Setipinnaphasa</i>	Rivers, estuaries	10	01	10%
<i>Latescalacarifer</i>	Coastal water, estuaries	28	06	21.42%
<i>Mugil cephalus</i>	Coastal water, estuaries	8	01	12.5%
<i>Pampus argenteus</i>	Marine & costal	12	02	16.66%
<i>Gudusia chapra</i>	River, ponds	16	02	12.5%
<i>Labeobata</i>	Ponds, tanks, rivers	12	0	0
<i>Channa punctata</i>	Ponds, tank, flood plain wetlands	9	01	11.11%
<i>Channa striata</i>	Ponds, tanks, flood plain wetlands	7	0	0%
Total		200	51	25.5%

Fig.1 Electrophoresis of 16S rDNA PCR products from *Vibrio parahaemolyticus*, isolated from fish and shell fish samples. Lane M – Molecular weight marker (Sigma), Lane- 1 to 7 *Vibrio parahaemolyticus*

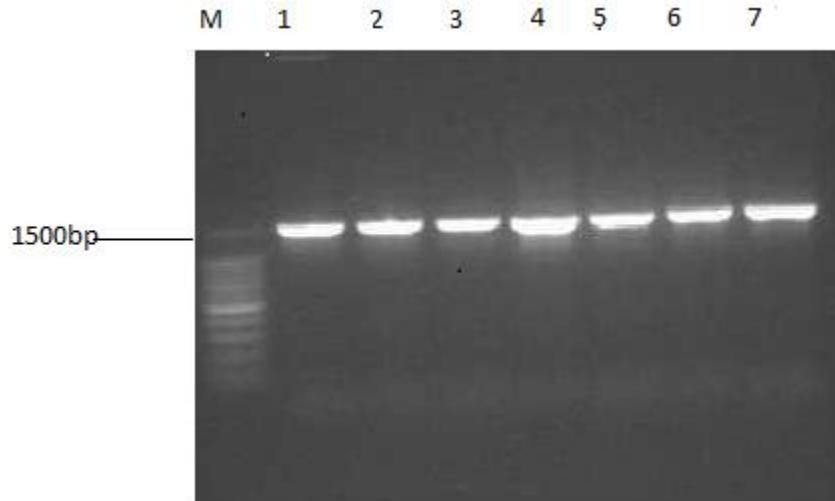


Fig.2 Phylogenetic tree showing the relationship of bacterial isolates (B2, B3, B4, B6 and B11 with type strains of *Vibrio parahaemolyticus*. B2 strain (KUHWB26)B3 strain (KUGWB43), B4 (KUBWB12), B6(KUBWB76)]

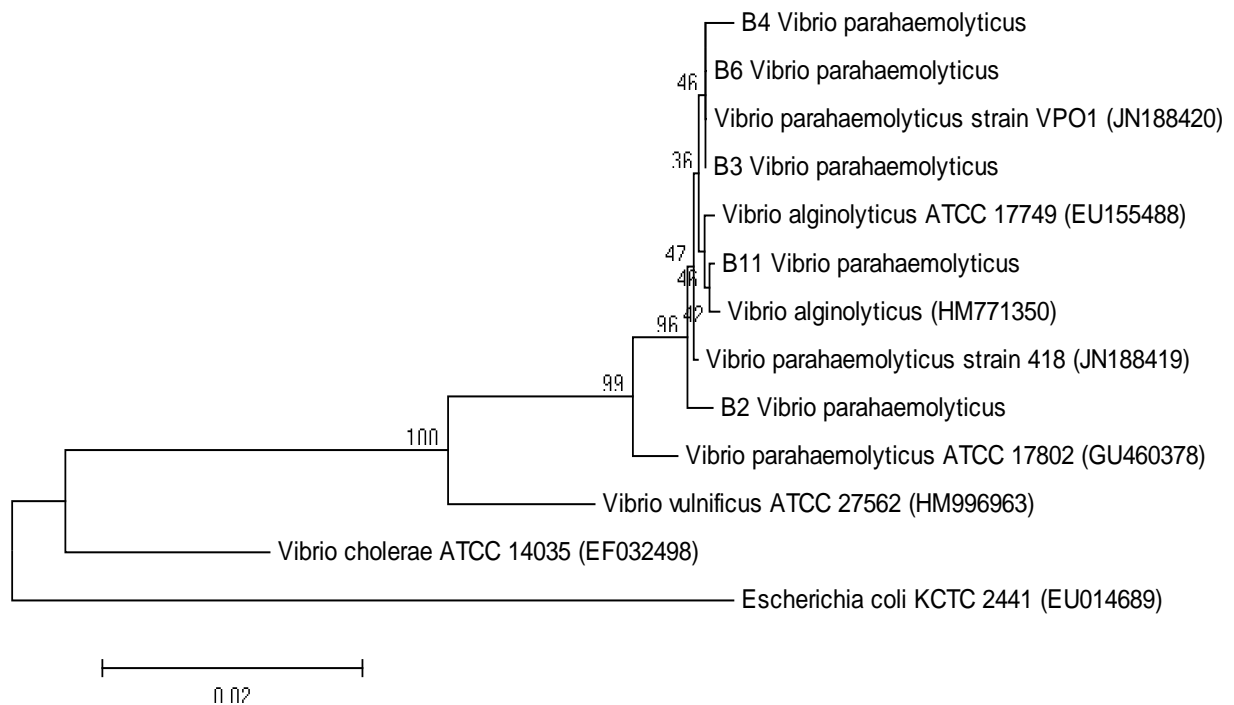
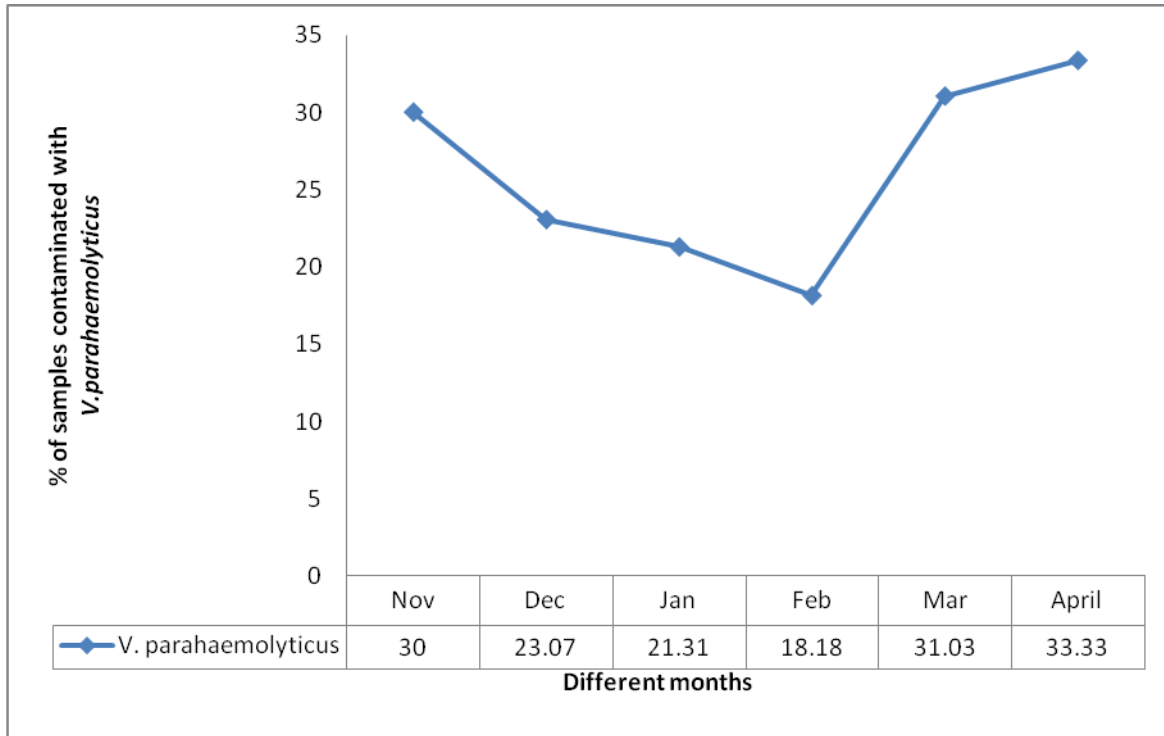


Fig.3 Prevalence of *V. parahaemolyticus* in fish and shellfishes in different months



Cross-contamination with *V. parahaemolyticus* of other fish from contaminated seafood during preservation, transport and handling at market place are envisaged as major sources of *V. parahaemolyticus* infection in India. Besides seafood, freshwater fishes have also been studied for *V. parahaemolyticus* contamination. It is known that most strains of *V. parahaemolyticus* isolated from the environment or seafood are not pathogenic to humans. Only a small portion (1-5%) of *V. parahaemolyticus* carrying the virulence genes encoding TDH and/or TRH can cause foodborne illnesses in humans (Hervio-Health *et al.*, 2002). Therefore, consumer needs to increase awareness and to ensure that the fish and shrimps are cooked properly and maintain sanitary hygiene. The epidemiology of *V. parahaemolyticus* infection has changed in India with emergence of pandemic clone of O3: K6 serotype (Bisha *et al.*, 2012). So, there is a high probability that the *V. parahaemolyticus* present fish and shellfish

samples may pose risk to human health through under cooked food and cross contamination of other food items, household refrigerators used for fish and shellfish preservation and kitchen environment.

The epidemiology and transmission of infection associated with *V. parahaemolyticus* are more prominent in Kolkata, India than other parts of the globe because people never eat raw fish and usually prefer fresh water fish over marine fish. The fresh water fish might be contaminated with *V. parahaemolyticus* by marine fish at market and cross contamination are considered as to be possible route of transmission in this setting (Nair *et al.*, 2007). The higher incidence of *V. parahaemolyticus* in shellfish could be correlated with the ability of the bacterium to utilize chitin, abundantly available in the crustaceans. Moreover, shrimps are rich in free amino acid content, which serves as an excellent growth medium for the proliferation of this bacterium

(Chakraborty *et al.*, 2008). However, salt preference of bacterium was also reflected due to its association in shrimps and various marine fin fishes in the study.

Seasonal variations in occurrence of *V. parahaemolyticus* was studied and it was observed that 20.85 % of finfish and shellfish collected during winter (December to February) were more contaminated by the bacterium in comparison to 31.45% of samples collected during summer months (November, March and April). The relationship between water temperature and prevalence of *V. parahaemolyticus* have been well studied by several researchers and higher occurrence was noticed during warmer seasons due to its proliferation in the aquatic environments (Nair *et al.*, 1980; Sarkar *et al.*, 1985; Daniels *et al.*, 2000; Charles-Hernandez *et al.*, 2006) which might have favored most of the outbreaks occur during warmer months. India being a tropical country is thus more prone to food-borne infections from the bacterium.

The present study has given an indication of hygienic and microbiological quality of fin fishes and shellfishes of domestic markets of West Bengal. Although, overall microbial load was within permissible limits, fishes were contaminated with *V. parahaemolyticus* raising food safety concern to the domestic consumers.

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