Abstract. – *Vibrio parahaemolyticus* is a marine bacterium which is also responsible for acute diarrhoeal illness in human beings. Eating raw seafish or contaminated seafood is responsible for acute gastroenteritis. The aim of this study was to investigate the isolation, identification and molecular characterization of *Vibrio parahaemolyticus* from the fish samples in Kolkata, India.

Materials and Methods: In this study 90 fish samples were collected from 8 different market places in Kolkata, India. Fish samples collected were shrimp, prawn, bhetki, pamfret and hilsa. VP-toxR PCR was performed to confirm the presence of species specific toxR. *tdh* and *trh* genes PCR for detection of virulence genes were performed separately. GS-PCR was performed in *tdh*, *trh* gene positive strains to determine whether they belong to pandemic genotype. Serotyping was also done on the *tdh*, *trh* positive strains.

Results: Out of the 90 fish samples collected from different local fish markets 60 were positive for *Vibrio parahaemolyticus*. 21 (35%) out of 60 *Vibrio parahaemolyticus* isolates from fish samples harboured the *tdh* gene. 1 (1.7%) out of 60 *Vibrio parahaemolyticus* isolates from fish samples carried *trh* gene. Out of 22 isolates only 2 were positive for GS-PCR. O10:KUT was the serovar maximum isolated.

Conclusion: Considerable percentage of *Vibrio parahaemolyticus* carrying the virulence genes and pandemic genotype among fish in Kolkata indicates that there is potential reservoir in Kolkata and consumption of sea fish or contaminated fish might cause *Vibrio parahaemolyticus* mediated diarrhea in this region.

Key Words: *Vibrio parahaemolyticus*, Fish, Diarrhoea, Acute gastroenteritis.

Introduction

*Vibrio parahaemolyticus* is a marine bacterium which is also responsible for acute diarrhoeal illness in human beings. Eating raw seafish or contaminated seafood is responsible for acute gastroenteritis. *Vibrio parahaemolyticus* was first implicated in an outbreak of food poisoning in Japan in 1950. Etiological studies of acute diarrhoeal illness in Kolkata have shown that gastroenteritis caused by *Vibrio parahaemolyticus* ranks next to *Vibrio cholerae* in terms of incidence. About 10% of the cases of gastroenteritis in patients admitted to Infectious Diseases Hospital in Kolkata are due to *Vibrio parahaemolyticus*. *Vibrio parahaemolyticus* accounts for about 70% of gastroenteritis cases associated with seafood in Japan. Presence of *Vibrio parahaemolyticus* infection in seafish, fish products or in fresh water fishes is of public health importance. Though *Vibrio parahaemolyticus* infection is self limited infections, socioeconomic loss and rarely death are some of the problems. Immunocompromised patients may die due to consumption of contaminated raw seafish or under cooked fish products. Sometimes international travelers are also responsible for spreading infection from one country to another country. Fish markets, fish harvesting areas, vectors like flies, sea water and sometimes fresh water are the main sources by which this bacterium may spread to human. Multiplication of *Vibrio parahaemolyticus* is related to water temperature and season also.

The aim of this study was:

1. To isolate *Vibrio parahaemolyticus* from fishes purchased from markets in and around Kolkata.

Corresponding Author: Debaprasad Pal, MD; e-mail: debaprasadpal@hotmail.com
2. To identify the serotypes of *Vibrio parahaemolyticus* isolated in the study.
3. To determine the virulence genes *tdh* and *trh* by PCR from *Vibrio parahaemolyticus* strains.

**Materials and Methods**

In this study 90 fish samples were collected from 8 different market places in Kolkata, India. Fish samples collected were shrimp, prawn, bhetki, pamfret and hilsa. Each fish sample was placed in aseptic small plastic bag, labeled and sealed separately to avoid contamination. The samples were placed in sealed containers with ice cubes and transported to the laboratory. Processing of samples were initiated within 2-3 hours of sample collection. In all specimens swabs of gills and intestines were taken for analysis. The swabs were incubated in alkaline peptone water (APW), which contained 3% NaCl at pH 8.0 and incubated for 18 hours at 37°C. This APW was used to enrich the *Vibrio parahaemolyticus*. Isolation and identification of *Vibrio parahaemolyticus* was carried out as described in Bacteriological Analytical Manual of Food and Drug Administration (FDA 1992). A loopful of broth was placed on Thiosulfate Citrate Bile Salts Sucrose (TCBS) at 37°C. Next day the colonies were examined for typical bluish green colour. The typical non-sucrose fermenting green colonies were subjected to standard biochemical tests in Kaper's agar slant for alkali/acidic (K/A) reaction. The strains which produced K/A reaction were stored into nutrient agar stabs containing 3% NaCl for further confirmation and to carry out further molecular characterization. VP-*toxR* PCR was performed to confirm the presence of species specific *toxR*. Template DNA Preparation

A loopful of culture was plated on 3% NaCl Luria Agar (LA) plate and kept for overnight incubation at 37°C. The small amount of overnight culture was taken with the help of toothpick and placed on sterilized microfuge tube containing 500 µl of triple distilled water. Homogenous suspension was made using vortex. These samples were kept in water bath adjusted at 98°C for 15 minutes to lyse the cells. After 15 minutes the culture lysate was snap cooled on ice for denaturation of template DNA. Amplification was carried out in an automated thermal cycler (Master cycler gradient, Eppendorf) using PrimerF 1781 and PrimerR 1782. Reference strain VP-KxV138 was included in PCR assay as a positive control. The amplified PCR products were kept at -20°C until loading into 2% agarose gel. 5-10 µl of the PCR product were mixed with gel-loading dye. In the first well a molecular weight marker (100 bp DNA ladder, Takara, Japan) was loaded. The reference strain PCR product was loaded in the second well. The documentation was done using gel documentation system (Gel Doc 2000, Bio-Rad, Ann Arbor, Michigan, USA). The authenticity of 368 bp amplicon of the *toxR* gene was confirmed by comparing with the position of the PCR product of the control strain (VP-Kx V138). *tdh* and *trh* genes PCR for detection of virulence genes were performed separately. The Vp-*toxR* positive strain culture lysates were used as template DNA.

GS-PCR was performed in *tdh, trh* gene positive strains to determine whether they belong to pandemic genotype of *Vibrio parahaemolyticus* by detecting *toxRS* sequence according to method of Matsumoto et al. Identification of serotypes of *Vibrio parahaemolyticus*

Currently 13 O and 72 K type of commercial antisera are recognized. Serotyping was performed on *tdh* and *trh* positive isolates of *Vibrio parahaemolyticus* by slide agglutination technique using commercially available antisera kits (Denka Seiken Ltd., Tokyo, Japan). Results

Out of the 90 fish samples collected from different local fish markets 60 were positive for *Vibrio parahaemolyticus*. The results of *V-toxR* confirmed 60 isolates as *Vibrio parahaemolyticus*. All the 60 isolates of *Vibrio parahaemolyticus* gave a 368 bp amplicon, which matched with the control strain VP-Kx138. Isolation of *Vibrio parahaemolyticus* from prawn, pamfret, bhetki and hilsa are 67.35%, 50%, 69.23% and 100% respectively. 21 (35%) out of 60 *Vibrio parahaemolyticus* isolates from fish samples harboured the *tdh* gene. All the 21 isolates of *Vibrio parahaemolyticus* gave a specific 199 bp amplicon in the *tdh* PCR which are comparable with the amplicon from the reference strain VP-Kx138. In the present study 1 (1.7%) out of 60 *Vibrio parahaemolyticus* isolates from fish samples car-
ried trh gene. The trh positive strain gave a specific 249 bp amplicon from the reference strain VP-V287 (Table I). Distribution of virulence genes among prawn, pamfret, bhetki, hilsa are 42.42%, 40%, 33.33% and 12.5% respectively. The tdh, trh gene positive isolates of Vibrio parahaemolyticus from fish samples were selected for group specific PCR. Out of 22 isolates only 2 were positive for GS-PCR that produced 651 bp amplicon. This result was comparable to reference strain VP-KxV_{224}. Percentage of Vibrio parahaemolyticus harboring tdh, trh and pandemic strains among fish isolates are 35%, 1.7% and 3.3% respectively. The tdh and trh gene positive isolates of Vibrio parahaemolyticus from fish sample were selected for serology. In this study O1:KUT serovar was identified with 2 isolates, O10:KUT serovar with 4 isolates and O1:K33 with 2 isolates (Table II).

### Discussion

Vibrio parahaemolyticus is a marine bacterium that occurs naturally in shellfish, oyster, prawn, crab, raw fish, blue mussels, salted herring, roe, fresh water fish, sea fish, etc. Fish is a good protein source and Kolkata is one of the places in India where consumption is very high. In Kolkata some of the fish variety is delivered from other states of India. Moreover this bacterium is commonly found in other South East Asian countries. In India the first outbreak of Vibrio parahaemolyticus mediated diarrhea was reported in Vellore, Tamilnadu^{15}. In 1996 an abrupt increase in diarrhoeal cases were reported in Infectious Disease Hospital, Kolkata due to pandemic strain O3:K6^{16}. PCR used to identify VPtoxR gene is species specific for Vibrio parahaemolyticus^{11}. As both pathogenic and nonpathogenic strains of Vibrio parahaemolyticus exist in seafood, PCR specific for virulence genes (tdh, trh) will help in detection of the pathogenic strains^{12}. In the present study, 66.7% isolates were positive for the Vibrio parahaemolyticus, VP-toxR confirmed 94% species specific tox-R gene and 22 (36.7%) out of 60 isolates were positive for virulence genes such as tdh or trh. Early works of Sakazaki et al^{17} reported that 1-2% of environmental samples contain virulent isolates. Recently Deepanjali et al^{8} reported that the environmental samples (oysters) contain 93.87% Vibrio parahaemolyticus and most of them harbor trh (59.3%) and a few with tdh (6.1%) in South West coast of India. Presence of 35% tdh, 1.66% trh and two pandemic genotype isolates in this region, strongly suggest that fish (seafood, fresh water fish) produce a conducive environment for this pathogen, which not only support its survival but also to retain virulence genes. Normally estuarine or sea water fish carries the Vibrio parahaemolyticus. In this study fresh water Hilsa collected from Kolkata were contaminated with pathogenic Vibrio parahaemolyticus. GS-PCR performed with 22 fish samples showed that 2 (3.33%) were positive for the 651 bp amplicon. GS-PCR has been utilized for detection of pandemic strains of Vibrio parahaemolyticus, utilizing nucleotide variation for ToxRS sequence. GS-PCR distinguishes new pandemic clones from old clones^{13}. GS-PCR positive strains belonged to O1:KUT is the serotype, which is responsible for increasing numbers of diarrhea among patients in as many as 12 countries^{8,19,20}. In contrast to Vibrio cholerae, Vibrio parahaemolyticus infection is not associated with exclusive serovar in the past. The O1:KUT and O8:KUT serovars isolated in this study belonged to pandemic genotype. The pandemic O3:K6 clone was isolated from environmental samples in Thailand^{21} and India^{8}. In vitro adherence and cytotoxicity studies with human epithelial cells showed that O3:K6 strains exhibited higher level of adherence and cytotoxicity to host cells than non O3:K6 strains contributing to unique pathogenic potential. Thermostable Di-
rect Haemolysin (TDH) and TDH related haemolysin (TRH), capsular polysaccharide (CPS), lipopolysaccharide (LPS) and serine protease are the four virulence factors playing an important role in pathogenicity of Vibrio parahaemolyticus infection.\textsuperscript{25,23} TDH is capable of producing $\beta$ haemolysis on Wagatsuma's agar which is called Kanagawa's phenomena.\textsuperscript{24} Over 90% of TDH produced by Kanagawa positive strains are attributed to tdh-2 and tdh-1 gene direct the synthesis of less than 10% of total TDH. TRH is another virulence factor has been discovered in clinical strains lacking tdh gene.\textsuperscript{25} TRH is a 23kDa protein, immunologically related to TDH and environmental strains producing this gene also produces urease. Considerable percentage of Vibrio parahaemolyticus carrying the virulence genes and pandemic genotype among fish in Kolkata indicates that there is potential reservoir in Kolkata and consumption of sea fish or contaminated fish might cause Vibrio parahaemolyticus mediated diarrhea in humans.

**References**

Isolation, identification and molecular characterization of *Vibrio parahaemolyticus*


Acknowledgements

The Authors wish to express their sincere thanks to Dr. M. Saravanan, Student MVPH for performing the laboratory techniques and to Dr. T. Ramamurthy, Deputy Director, Division of Molecular Microbiology, National Institute of Cholera and Enteric Diseases, Kolkata for helping to do the PCR in his Laboratory.