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Original Article

Seasonal Variation and Molecular Characterization of *Vibrio parahaemolyticus* Isolated from Karnaphuni River and Estuary of Chittagong, Bangladesh

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ABSTRACT: To determine whether the environmental variables governing the dynamics of *Vibrio parahaemolyticus* populations, sediment samples were collected with or without 1% NaCl from the Karnaphuli River and Karnaphuli Estuary of Chittagong, Bangladesh. The MPN method was used for quantitative analysis of the organisms. The isolates were subjected to biochemical tests for confirmation and also for the molecular characterization including assessment of virulence properties. Higher occurrence of *V. parahaemolyticus* was observed during periods of lower salinity. Thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) are considered as major virulence factors for the organism and are coded by the *tdh* and *trh* genes, respectively. This study was, therefore, carried out to observe the seasonal variation and to investigate the occurrence of *tdh* and *trh* containing *V. parahaemolyticus*, besides the brackish environments. Twelve isolates out of 15 showed identical patterns to biochemical test profiles of the reference strain. This was further confirmed by PCR using primers specific for *toxR* gene. Antibiogram using nine commercial antibiotics showed that these isolates were sensitive to all representative antibiotics except ampicillin and streptomycin. All *V. parahaemolyticus* isolates were found negative for *tdh* gene which was confirmed by both reverse passive latex agglutination test and PCR. The most important finding of the study was the regular occurrence of *V. parahaemolyticus* in river of 0% salinity, though most of them are nonpathogenic strains. However, they could not grow in nutrient agar media having no NaCl.

Keywords: *Vibrio parahaemolyticus*, Seasonal Variation, Thermostable direct hemolysin-TDH, TDH related hemolysin-TRH, Antibiogram.

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INTRODUCTION

Vibrio parahaemolyticus, a well-known human pathogen, is the leading cause of gastroenteritis due to the consumption of seafood, primarily raw or improperly cooked shellfish worldwide (Tison and Kelly, 1984). This organism is considered to be restricted to a saline environment, and it requires Na^+ for survival and growth, but several studies suggest it also occurs in brackish water (Bockemühl *et al*., 1986), plankton, and fish (Sarkar *et al*., 1985) of freshwater environments. This bacterium causes approximately half of the all food poisoning cases in Taiwan, Japan and several Southeast Asian countries

(Joseph *et al*., 1982, Chiou *et al*., 1991). It is also reported to be an important agent of travelers' diarrhoea and has also occasionally been associated with extra intestinal infections, including wounds (Blake *et al*., 1980). Serotyping can differentiate isolates of *V. parahaemolyticus* and 13 O types and 71 K types have been identified (Iguchi *et al*., 1995). Since 1996, an increased incidence of gastroenteritis in many parts of the world has been associated with *V. parahaemolyticus* serotype O3:K6 (Chiou *et al*., 1991, Okuda *et al*., 1997). The association of O3:K6 serotype with large-scale food-borne disease outbreaks in Taiwan, Laos, Japan, Thailand, Korea and the United States between 1997 and 1998 suggest this organism may have an unusual capacity to be transmitted by foods and/or to cause human infection and has pandemic potential (Matsumoto *et al*.,2000). Since 1998, *V. parahaemolyticus* strains belonging to other two serotypes, O4:K68 and O1:KUT (UT indicates untypable), have also been isolated with increasing frequency from diarrheal patients (Chowdhury *et al*., 2000). Pathogenic strains of *V. parahaemolyticus* generally produce a thermostable direct haemolysin (TDH) that is associated with the Kanagawa phenomenon (KP) and/or thermostable direct haemolysin-related haemolysin (TRH). Both TDH and TRH encoded by *tdh* and *trh* genes respectively are now recognized as major virulence factors in the pathogenesis of *V. parahaemolyticus* (Honda and Iida, 1993, Nishibuchi and Kaper, 1995). A separate gene, Thermolabile haemolysin (*tlh*) has also been characterized (Taniguchi *et al*., 1986). This gene was shown to be present in all of the *V. parahaemolyticus* strains tested previously (Taniguchi *et al*., 1986). The pathogenicity of *V. parahaemolyticus* isolates has traditionally been correlated with the production of the thermostable direct haemolysin (Vp-TDH) which is responsible for the beta-haemolysis observed when the organisms are plated on a modified blood agar known as Wagatsuma agar (Chun *et al*., 1975). More than 90% of clinical *V. parahaemolyticus* isolates but less than 1% of food or environmental strains produce TDH or possess *tdh* (DePaola *et al*., 2000). Although *V. parahaemolyticus* has been thought to be a marine bacterium because of its indispensable Na^+ requirement for survival and growth, the occurrence of this moderately halophilic organism in fresh water has also been observed (Venkateswaran *et al*., 1989). These evidence may suggest that the habitats of V. *parahaemolyticus* is somewhere out of marine environment which inspired us to turn our attention towards the rivers of Bangladesh besides the estuarine environments. The aim of this study was therefore to investigate the occurrence of *V. parahaemolyticus* in the estuaries as well as in the rivers of Bangladesh and to determine seasonal variation, the phenotypic and genotypic traits of these *V. parahaemolyticus* strains isolated from the sediment samples of Karnaphuli river and also from an estuary in the Chittagong near the Bay of Bengal by biochemical characterization, tolerance of NaCl concentration, serotyping, antibiogram, followed by PCR for *toxR*, *tdh*, and *trh* and Reverse Passive Latex Agglutination for toxin detection. Thus this study becomes significant as it presents the molecular characterization of the environmental *V. parahaemolyticus* isolates of these regions that may be responsible for diarrheal cases occurring sporadically.

MATERIALS AND METHODS

Sampling sites

Sampling was done from two sampling sites of different aquatic bodies in Karnafuli River and Estuary located in Chittagong district from January 2008 to August 2008.

Collection of environmental samples

Sediment samples from two different water bodies in Karnaphuli River and Karnaphuli Estuary were collected at 15-day intervals from January 2008 to August 2008. Therefore, a total of 96 samples were collected during the time period. Sediment samples were collected from 10 cm depth of the shore. Several grams of samples were aseptically collected by wearing sterile gloves to sterile plastic bags having 1% salt and another set was without salt. Samples were stored at room temperature until processing in the laboratory.

Cultural properties on X-VP plate and counting

Morphological characteristics of colonies developed after incubation on X-VP agar plate were carefully studied and recorded. Following overnight incubation at 37°C, blue-green colonies on X-VP agar plates were selected as suspected *V. parahaemolyticus*. They were counted as drop plate method. Five to ten suspected *V. parahaemolyticus* colonies were picked up from the X-VP agar plate and sub cultured onto fresh X-VP plate for pure culture.

Screening of suspected isolates on TCBS plate

Suspected *V. parahaemolyticus* isolates from the fresh X-VP plate were streaked onto thiosulphate citrate bile salt sucrose (TCBS) agar (TCBS agar; Nissui Co., Japan) plate for presumptive confirmation as *V. parahaemolyticus*. Following overnight incubation at 37°C, isolates that produced mucoid, raised, green colonies on TCBS agar plates were selected as *V. parahaemolyticus*. The selected isolates were preserved in preservation medium then subjected to biochemical identification and other molecular based assays.

Reference strains

V. parahaemolyticus strains of VP11 (new clone of serotype O3:K6, harboring tdh derived from a patient in Aomori Prefecture in Japan in 2000) and AQ3857 (serotype O1:K1, harboring both tdh and trh isolated from international traveler in Hong Kong in 1983) were used as reference strains to compare the

biochemical characteristics and gene analysis. Biochemical, serological and molecular tests according to the standard procedure reconfirmed the strains.

Biochemical identification

After incubation, characteristic colonies on TCBS agar plates selected as *V. parahaemolyticus* were subjected to the following standard biochemical tests in order to confirm the isolates as *V. parahaemolyticus*. Biochemical tests were performed according to the methods described in Microbiology Laboratory Manual (Cappuccino and Sherman, 1992). The biochemical tests were Kligler's iron agar test, lysine decarboxylase test, oxidase test, citrate utilization test, motility test, ornithine decarboxylase test, indole production test, methyl-red test, Voges-Proskauer test and salt tolerance test.

Pure culture preparation

Pure cultures of test organisms were obtained by sub culturing on LB agar with 3% NaCl plates after incubation at 37°C for 18-24 h.

Salt tolerance test

All isolates were tested for their salt tolerance in alkaline peptone agar (APA) plate containing 0%, 3%, 7% and 10% (w/v) sodium chloride. Fresh culture was streaked onto an APA plate and growth was observed visually after 24 h. of incubation at 37°C.

Detection of *toxR* gene by PCR

Isolates confirmed by the biochemical tests were further analyzed for the presence of *toxR* gene of *V. parahaemolyticus* by PCR using primers specific for toxR.

Preparation of template DNA

Each isolate of *V. parahaemolyticus* was cultured in LB broth containing 3% NaCl and was incubated at 37°C for overnight. The cell pellet was harvested by centrifugation of 1 ml of culture at 10,000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in phosphate buffered saline (PBS), and then washed by centrifugation at 10,000 rpm for 3 minutes. This procedure was repeated for two times. The pellet was re-suspended in TE buffer solution and boiled for 10 minutes. Then it was put immediately into ice for minimum 30 minutes. After that centrifuged at 13,000 rpm for 10 minutes on a tabletop centrifuge (Eppendorf centrifuge, 5804, Germany) and the supernatant was collected. The supernatant was used as the template solution for PCR. The template DNA was then mixed with reaction mixture.

Post PCR detection of amplified DNA by electrophoretic analysis

The successful amplification of the *toxR* gene was examined by resolving the PCR products in 1.5% agarose gel. 0.75g agarose (Sigma) was dissolved in 50 ml of 1X Tris-borate EDTA (TBE) buffer to give a final concentration of 1.5% agarose and was heated to dissolve in a microwave oven (Model: D90N30 ATP, Butterfly, China) for about 2.5 - 3 min. 1.5 µl of 10 mg/ml ethidium bromide was mixed with the molten agarose. When the temperature came down to 50°C, the gel was poured onto the gel tray already fixed with appropriate combs. Following solidification of the gel, it was submerged in 1 X TBE buffer in a gel running tank. 8 µl PCR product was mixed with 2 µl of 10X gel loading dye and loaded into the slots of the gel with the aid of a micropipette. Electrophoresis was continued with 60 volts until DNA fragments were separated. The EtBr stained DNA bands were observed on a UV transilluminator (Model TS-40, USA). Photographs were taken using Gel Documentation machine attached to a computer and bands were analyzed with "Quality one" software (Bio-Rad, USA). The PCR products sizes were estimated using the 100 bp marker (Invitrogen, USA)

tdh Toxin Assay by Reverse Passive Latex Agglutination Test (RPLA)

tdh toxin assay was done using a commercially available Reverse Passive Latex Agglutination (RPLA) kit (Deneke and Colwell, 1973)(Denka Seiken, Tokyo, Japan)

Detection of *tdh* and *trh* virulence gene by PCR

Isolates showing positive results for *toxR* gene by PCR were further analyzed for the presence of virulence associated gene *tdh* and *trh* of *V. parahaemolyticus* by PCR using primers specific for *tdh.*

Electrophoretic analysis

The PCR amplified mixture was resolved by electrophoresis in a 1.5% agarose gel to detect the amplicons following the method already described in above section.

Determination of Antibiotic Susceptibility of the test isolates

Susceptibility of each test isolate to different antibiotics was determined *in vitro* by employing the modified Kirby-Bauer (Bauer *et al.*, 1966) method. The zone diameters for individual antimicrobial agent were then translated into susceptible, intermediate, moderately susceptible, or resistant categories according to the interpretation table (Standards and Watts, 1999).

RESULTS

Primary isolation of *V. parahaemolyticus* from environment

Alkaline peptone water (APW) enriched environmental samples were drop plate on X-VP agar medium were incubated for 24 hours at 37°C. Then observed for the presence of *V. parahaemolyticus* colonies. On the X-VP agar medium, the suspected *V. parahaemolyticus* were found to appear as characteristic blue green colonies and count them by MPN method. Count expressed in log cfu/gm shown in **figure 3.3** in different period of different water bodies. Of them only four colonies were transferred to TCBS agar plate of each sampling. On the TCBS agar plates,

the suspected *V. parahaemolyticus* were found to be large, green, raised and mucoid colonies of approximately 2-3 mm in diameter. These colonies were then preserved for further experiment in stock culture.

Seasonal variation of *V. parahaemolyticus* in estuary and river environment

V. parahaemolyticus is a halophilic bacterium that may cause serious human infection isolated from fresh water of 0% salinity. The growth of *V.parahemolyticus* is directly related to environmental factors. These factors are mainly salinity and temperature. So they varied from season to season on the basis environmental conditions. The environmental parameters for the seasonal variation in this study at the two sampling sites are discussed below:

Effect of temperature

Environmental samples were collected from January, 2008 to August, 2008 were analyzed for physical characteristics and the presence of *V. parahaemolyticus* to assess seasonal effects on the occurrence of the organism. Temperatures of Karnaphuli river and estuary were recorded during study period. Water temperatures varied widely over the year, ranging from 19°C in February to a high of 30°C in August. The growth of the organisms was lower in low temperatures. Recovery of the organisms increased gradually through the summer. The seasonal variation of temperatures in Karnaphuli river and estuary are shown in the **figure 3.1.**

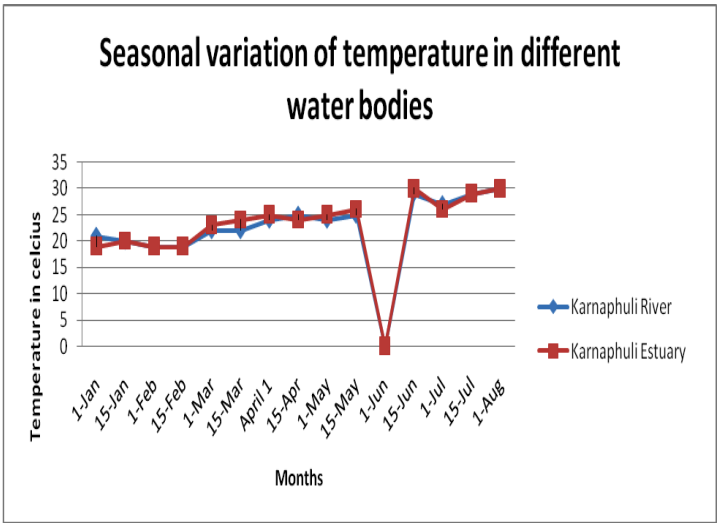


Figure 3.1. Seasonal variation of temperature (in Celsius) different water bodies.**(No Sampling in 1 June)**

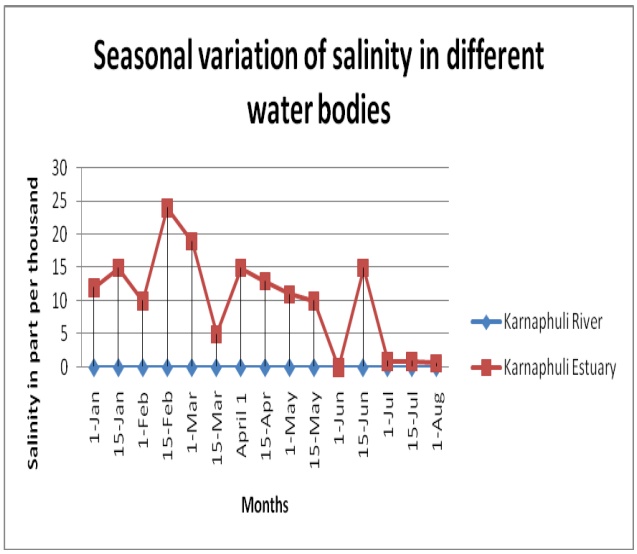


Figure 3.2. Seasonal variation of salinity of different water bodies. **(No sampling in 1 June)**

Effect of salinity

Salinity of the study areas were recorded routinely and it was varied from 0.7‰ to 24‰ in the estuary of Karnaphuli but 0‰ in the Karnaphuli River. Seasonal variation of salinity in environment is shown following **figure 3.2**. Seasonal variation of *V. parahemolyticus* is stated in the **figure 3.3**. There was a regular occurrence of *V. parahaemolyticus* in the river and estuary. Counts were expressed in log cfu/gm. A comparative study of river and estuary on the basis variation of temperatures, salinity and counts are shown in **Figure 3.4 and 3.5**.

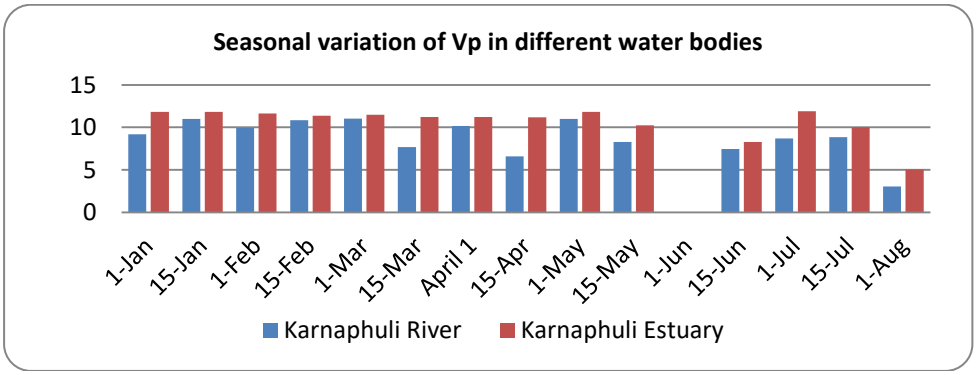


Figure 3.3. Seasonal variation of Vp in different water bodies. Count in Log of cfu/gm

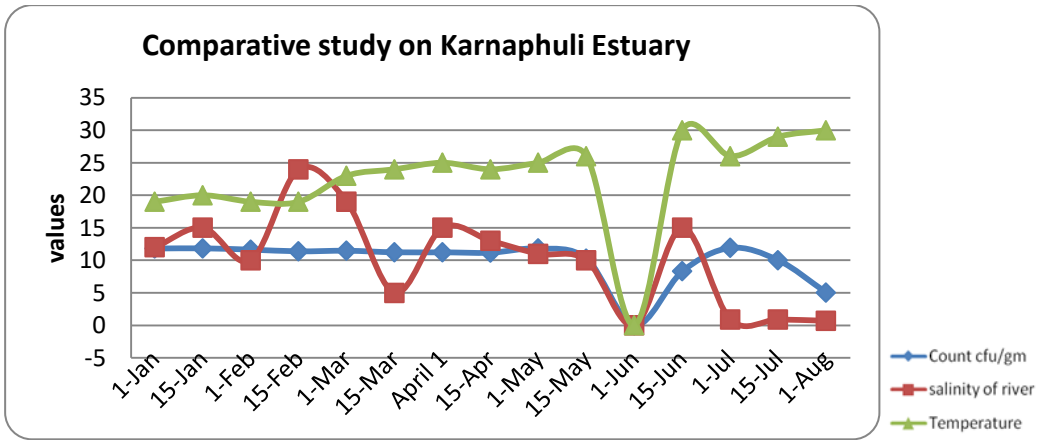


Figure 3.4. Comparative study on Karnaphuli Estuary

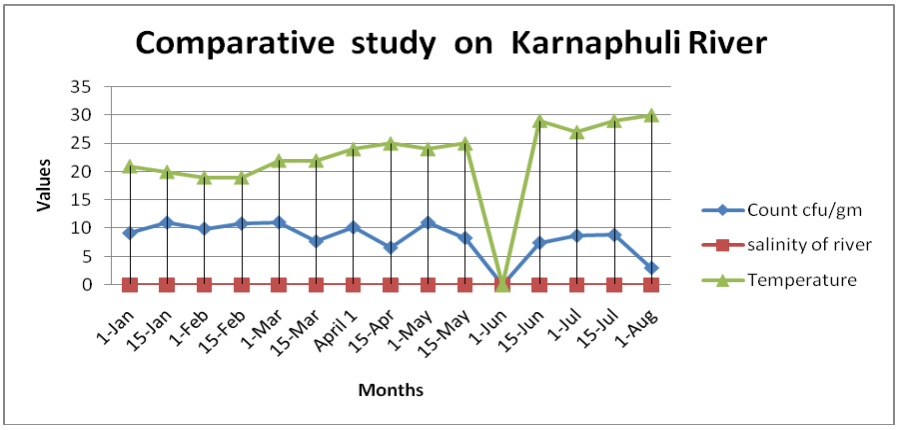


Figure 3.5. Comparative study on Karnaphuli River

Biochemical identification

Stock cultures of *V. parahaemolyticus* were subjected to extensive biochemical tests. Isolates that showed positive reactions to oxidase test, citrate utilization test, methyl red test, lysine and ornithine decarboxylases, that demonstrated alkaline red slant and acidic yellow butt in KIA test, indole production, motility, that could tolerate 3%, and 7% NaCl at 37°C that gave negative reaction to Voges-proskauer, and couldn't grow in 0% and 10% NaCl were identified as *V. parahaemolyticus*. Results of biochemical reactions of the 15 isolates from rivers and estuary shown in table **3.1**

Table 3.1. Results showing biochemical reactions of the 15 isolates from rivers and estuary

Area of isolation	Strain ID	KIA			Oxidas	Lysine	Ornithi	Motilit	Indole	Citrate	MR	VP	NaCl %			
		Slan	Butt	H2S									0	3	7	10
Karnaphuli (River)	**K1**	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-
	K2	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-
	K3	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-
	K4	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-
	K5	K	A	-	+	+	+	+	-	+	-	-	-	+	+	-
	K6	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-
	K7	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-
	K8	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-
Karnaphuli (Estuary)	**E1**	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-
	E2	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-
	E3	K	A	-	+	+	+	+	-	+	-	-	-	+	+	-
	E4	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-
	E5	K	A	-	+	+	+	+	+	+	-	-	-	+	+	-
	E6	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-
	E7	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-
Positive control	**VP 11**	**K**	**A**	-	+	+	+	+	+	+	+	-	-	+	+	-

[Keys: K= Alkaline; A= Acidic; + = Positive; - = Negative]

Among 15 isolates, 12 isolates (7 River and 5 estuary isolates) (shown in bold) gave all characteristic biochemical reactions and hence were identified as *V. parahaemolyticus*.

Reference strain VP11 (new clone of serotype O3:K6) used as positive control in these biochemical tests.

Detection of *toxR* gene of *V. parahaemolyticus* by PCR

All isolates showing positive biochemical results were also observed for the presence or absence of *toxR* gene, using *tox*R specific primers; since *tox*R gene appears to be well conserved among *vibrio* species and its regulatory function in *V. parahaemolyticus* has already been described in many reports. when template DNA from each isolate was subjected to amplification through PCR, all isolates yielded DNA band (399bp) specific for *tox*R region after 35 cycles of amplification followed by agarose gel electrophoresis **(Figure 3.6)**

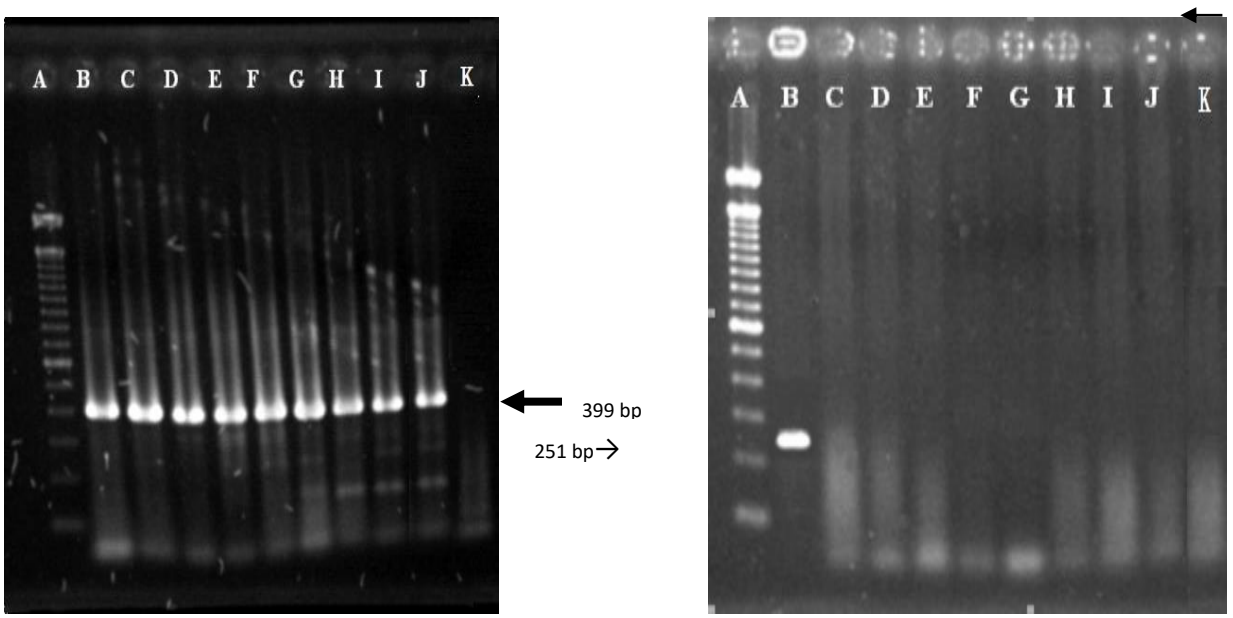
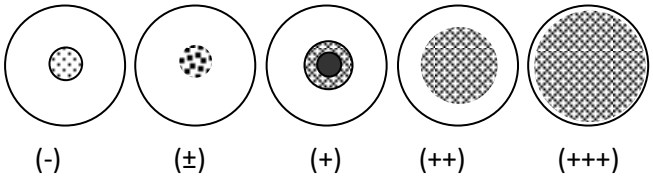


Figure 3.6, 3.7. Agarose gel electrophoresis pattern of PCR amplicons of isolates from estuary and river obtained with primers specific for *toxR* and *tdh* gene.

Key: Lane A-100bp molecular weight marker.
Lane B- Positive Control strain VP11 (New clone of serotype O3:K6)(399 & 251 bp)
Lane C to J- Test isolates from estuarine and river sediment sample
Lane K-Negative control

***tdh* Toxin assay by Reverse Passive Latex Agglutination Test (RPLA)**

The agglutination pattern judged by comparison with the following illustration:



Results classified as (+), (++) and (+++) are considered to be positive. Results classified as (±) and (-) are considered to be negative. Results for RPLA toxin assay of *V. parahaemolyticus* are presented in **Table 3.2**

Table 3.2: RPLA toxin assay of 12 isolated strains

Sample	Strain ID	Result (Toxin production)
Karnaphuli (River)	K1	-
	K2	-
	K3	-
	K4	-
	K6	-
	K7	-
	K8	-
Karnaphuli (Estuary)	E1	-
	E2	-
	E6	-
	E7	-
	E8	-
Positive Control	VP11	+

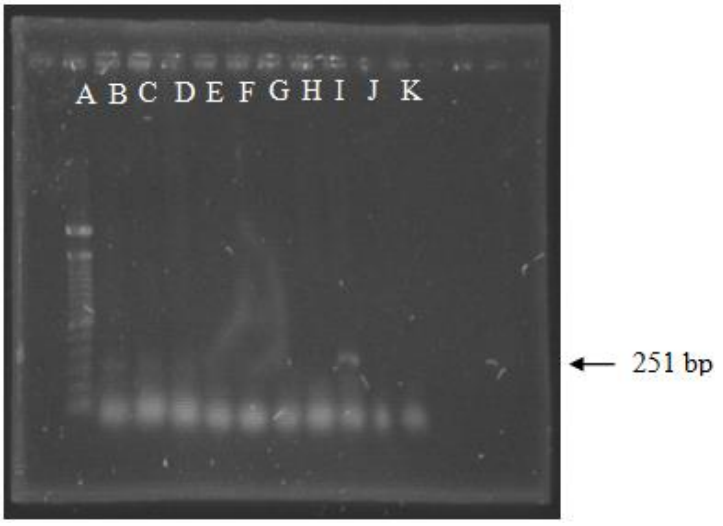


Figure 3.8. Agarose gel electrophoresis pattern of PCR amplicons obtained with primers specific for *trh*
Key: Lane A-100 bp molecular weight marker.
Lane B-Positive control strain VP11 (New clone of serotype O3:K6)
Lane C to J- Test isolates from river and estuarine sediment
Lane K- Negative control.

Detection of *tdh* virulence gene *V. parahaemolyticus* by PCR

V. parahaemolyticus isolates which yielded DNA bands (399bp) specific for *toxR* were further subjected to PCR using *tdh*- specific primers as a reconfirmatory test for the presence of virulence gene *tdh*. All the isolates showed negative results for the presence of *tdh* gene after 35 cycles of amplification.

An amplified fragment of 251 bp was detected after agarose gel electrophoresis for positive control **(Figure 3.7)**

Detection of *trh* virulence gene of *V. parahaemolyticus* by PCR

All *V. parahaemolyticus* isolates which yielded DNA bands (399 bp) specific for *tox*R were also subjected to PCR using *trh*- specific (251bp) primers as a reconfirmatory test for the presence of virulence gene *trh*. All the isolates showed negative results for the presence of *tdh* gene after 35 cycles of amplification. **(Figure 3.8)**

Antibiogram

Is this study, all the 12 isolates together were examined for their susceptibilities against the nine commonly prescribed antibiotics by the disc diffusion method (Bauer *et al.*, 1966) and the following results were obtained. The susceptibility pattern of the test isolates has been shown in **Table 3.3** below.

Table 3.3. Drug Resistance Pattern of *V. parahaemolyticus*

Sample	Strain ID	Susceptibility Pattern								
		AMP	TE	CN	CIP	E	SXT	C	S	K
KARNAPHULI (River)	K1	R	S	S	S	I	S	S	R	I
	K2	R	S	S	S	I	S	S	I	I
	K3	R	S	S	S	I	S	S	R	I
	K4	R	S	S	S	I	S	S	R	R
	K6	R	S	S	S	I	S	S	I	I
	K7	S	S	S	S	I	S	S	R	I
	K8	R	I	S	S	I	S	S	R	I
KARNAPHULI Estuary	E1	R	S	S	S	I	S	S	S	I
	E2	R	S	S	S	I	S	S	S	I
	E6	R	S	S	S	I	S	S	S	I
	E7	R	I	S	S	I	S	S	S	I
	E8	R	I	S	S	I	S	S	S	I
Positive control(O3:K6)	VP11	R	S	S	S	I	S	S	S	I

Key: AMP-Ampicillin (10µg), TE-Tetracycline (30µg), CN-Gentamycine (10µg), CIP-Ciprofloxacine(5µg),SXT-Sulphamethaxozole/trimethoprim(25µg), C- Chloramphenicol (30µg), S-Streptomycin(10µg), K-Kanamycin(30µg) R-Resistant, S-Sensitive, I-Intermediate.

Susceptibility of the test isolates were determined according to the reference zone sizes against the respective antibiotics. All the *V. parahaemolyticus* isolates were found susceptible to Sulphamethaxozole**,** tetracycline and ciprofloxacin. For gentamycin some isolates showed intermediate susceptibility. However, most of them found resistant only to Ampicillin and streptomycin. Figure depicts their susceptibility pattern to different antibiotics.

DISCUSSION

By integrating various data obtained on the occurrence of *V. parahaemolyticus* in water and sediment samples of Karnaphuli River indicates a fairly clear idea on the occurrence and distribution of this organism in freshwater environment in Chittagong. The incidence of *V .parahaemolyticus* strains in freshwater ecosystems is primarily related to their association with a biological host, particularly fishes. *V. parahaemolyticus* distribution in water and sediments at the two sampling sites were very frequent and regular. The low salinity of freshwaters and the relatively oligotrophic nature of these niches are probably the factors limiting the distribution of this moderately halophilic organism in freshwater and freshwater sediments.

Isolation and identification of the target bacterium is very crucial for its characterization. Isolation of *V. parahaemolyticus* from natural environment requires a pre enrichment step followed by growth on selective media. The suspected *V. parahaemolyticus* strains were then identified very carefully by biochemical tests. Isolates that showed positive reactions to oxidase test, citrate utilization test, methyl red test, lysine and ornithine decarboxylases, that demonstrated alkaline red slant and acidic yellow butt in KIA test, indole production, motility, that could tolerate 3% and 7% NaCl at 37°C; that gave negative reaction to Voges Proskauer and could not grow in 0% and 10%NaCl were identified as *V. parahaemolyticus* .

Biochemically positive *V. parahaemolyticus* strains were then subjected to the molecular based assay Polymerase chain reaction (PCR). The presence of *toxR* gene was used for identification of *V.parahaemolyticus*. In this study, *tox*R gene sequence was used in a PCR method for the specific identification of *V.parahaemolyticus*. All the test isolates showed positive results in PCR using primers specific for *toxR* gene.

Thereafter molecular characterization of these *V. parahaemolyticus* isolates were carried out by detection of toxin production and presence of virulence genes-*tdh* and *trh*. To detect *tdh* toxin production by *V. parahaemolyticus* isolated from environmental samples, reverse passive latex agglutination test was carried out. In this technique, the sensitized latex particles agglutinate in the presence of *tdh* toxin produced by *V. parahaemolyticus* and results in the formation of *tdh* toxin. This finding deserves more characterization like detection of relevant gene producing this enzyme by PCR. From this point of view, PCR for *tdh* gene was carried out.

In this study, PCR had been done for identifying the distribution of *tdh* gene. Of all isolates, none gave positive results in PCR using primers specific to *tdh* gene. The findings correlates with the results obtained by reverse passive latex agglutination test. Further PCR analysis was done for the detection of another virulence factor *trh* gene. Among the test strains, no one was positive for *trh* gene and thus considered as avirulent.

As antibiotic susceptibility is an important parameter for the treatment measures and current study antibiogram profile of the test isolates were investigated. No significant difference was observed between test isolates and positive control O3:K6 with respect to antibiotic susceptibility. The isolates were susceptible to almost all the representative antibiotics tested including ciprofloxacine,tetracycline, kanamycine, erythromycin

sulphamethaxazole/trimethoprim,gentamycine which is a good indicator in the treatment of *V. parahaemolyticus* associated with diarrhea and gastroenteritis. They were resistant to only ampicillin. Few strains showed intermediate resistance to only gentamycine and some strains were resistant to streptomycin. None of the isolates showed multi- drug resistance markers like the plasmid, the class 1 integron and the SXT element. Therefore, antibiotic resistance-associated spread of the isolates seems unlikely and treating these strains with those antibiotics would be possible. Sulphamethoxazole/trimethoprim and tetracycline seemed to be most effective with highest zone diameter of growth inhibition when tested by disc diffusion assay.

The study was designed to observe the seasonal variation and characterize the isolated *V. parahaemolyticus* strains from river and estuary in Chittagong. The most important finding of the study was the regular occurrence of *V. parahaemolyticus* in river of 0% salinity though most of them are nonpathogenic strains. The findings of this study could be useful to compare the clonal variation between clinical and environmental isolates belonging to the same serogroup of *V. parahaemolyticus.*

CONCLUDING REMARKS

Vibrio. parahaemolyticus has been thought to be a marine bacterium because of its indispensable Na^+ requirement for survival and growth. But the occurrence of this moderately halophilic organism in fresh water has also been observed (Bockemühl *et al.*, 1986) which is quite alarming due to the undeniable dependence of people on fresh water. During collection, a set of 1% NaCl containing samples and another set without 1% NaCl used. Salt containing samples showed a regular count but the count of *V.parahaemolyticus* was random and infrequent in the samples without salt. The study was thus designed to observe the seasonal occurrence of *V. parahaemolyticus*in in the rivers of Bangladesh besides the estuarine environments and also characterize them at the molecular level. Therefore, Using 1% NaCl during sample collection for the isolation of *Vibrio parahaemolyticus* from fresh water without any salinity is an innovative procedure and the occurrence of *V. parahaemolyticus* in the river was found frequent and regular.

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