PHYSIOCHEMICAL CONDITIONS AND CONTAMINATION WITH VIBRIOS OF SURFACE WATER AT MATLAB, BANGLADESH

Debasish Paul[1], Zahid Hayat Mahmud[1], Md. Shafiqul Islam[1], Sucharit Basu Neogi[1], Md. Sirajul Islam[1] and Iqbal Kabir Jahid[1,2]

[1]Environmental Microbiology Laboratory, Laboratory Sciences Division, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), GPO Box-128, Dhaka-1000, Bangladesh;
[2]Department of Microbiology, Jessore Science and Technology University, Jessore-7408, Bangladesh

*Corresponding author: ikjahid@yahoo.com

ABSTRACT

Cholera is water-borne infectious disease caused by *Vibrio cholerae* O1 and O139. Currently a proportion of people living in rural area like Matlab, Bangladesh are dependent on surface water for various purposes to surface water. People of the area are affected by cholera particularly in rainy seasons. We measured *in situ* the physicochemical parameters of water using portable meters and counted the *Vibrio* sp. from water, phytoplankton and zooplankton samples of river and pond water of Matlab, Bangladesh. The culturable Vibrios were counted thiosulfate-citrate-bile salts-sucrose agar (TCBS) and taurocholate tellurite gelatin agar (TTGA) plates and viable but nonculturable (VBNC) by direct fluorescent microscopy. To confirm, we further did PCR of *omp*W gene and serology for *V. cholerae* O1/O139. Strong correlation of Vibrios cultural counts with pH, total coliform and faecal coliform heterotropic plate counts and viable but nonculturable (VBNC) counts with only total dissolved solids (TDS) noticed from the results. We identified 25 *V. cholerae* non-O1 and five *V. cholerae* O1 serotype which is main cause of cholera from water, phytoplankton and zooplankton samples of pond and rivers. Therefore, the present study indicated that the

surface water sources are the main causes of cholera and reestablished the Matlab as epidemic area of Bangladesh.

Keywords: *Vibrio*, physicochemical parameters, Bangladesh, biochemical test, *omp*W PCR

INTRODUCTION

Diarrhoea, the one of the most threatening diseases to date kill an about 2.5 million people each year, most of the children under 5 years (**Kosek *et al.*, 2003**). An estimated four billion cases annually account for 5.7% of the global burden of disease and place diarrhoeal disease as the third highest cause of morbidity and sixth highest cause of mortality (**WHO, 2006**). In Bangladesh 87% of total population live in the village of which, only 60% get the facilities of sanitary latrines (**GOB, 2003**).When people come by contact with contaminated water by bathing, washing or drinking, they may contact the disease. Mostly indicator organisms assess the degree of fecal pollution of fresh water since river, lakes and ground waters are common sources of community water supplies. Aquatic flora and fauna of marine environment as food where salt concentration is negligible is the cause of primary transmission (**Miller *et al.*, 1985**). Islam *et al.* (**1993**) showed that *Anabaena* sp. could provide a microenvironment for protected survival of *V. cholerae* O1 in both the microcosm and the aquatic environment of Bangladesh. These extra cellular products of blue-green algae may act as nutrients for *V. cholerae* (**Islam *et al.*, 1999**). All pathogenic *Vibrio* spp. secret an extra cellular chitinase and utilize chitin of copepods as a source of nutrient. A single copepod can harbor as many as 10^4 *V. cholerae* O1 cells (**Colwell, 2002**). Estuarine and freshwater environments both represent the critical reservoirs of *Vibrio* sp. (**Epstein *et al.*, 1993**). Pathogenic Vibrios correlate by the physicochemical features of the environment (**Epstein, 1993**). However, we know that a high bacterial density correlate with coastal eutrophication, and proliferation of various disease causing agents (**Epstein *et al.*, 1993**). Various physical and chemical parameters of the aquatic environment affect the physiological state and the pathogenic potential of *V. cholerae* (**Colwell *et al.*, 1990**). Islam *et al.* (**1990**) noted that *V. cholerae* increase their toxin production owing to selective pressure of physicochemical conditions in the aquatic environment. The cultivability of *V. cholerae* might be directly influenced by temperature, pH, and salinity (**Miller *et al.*, 1984**). Moreover, the number of *V. cholerae* could increase when plankton bloom in aquatic environments and cause cholera epidemics (**Islam *et al.*, 1994**). Increase in water pH is favorable for the growth of *V. cholerae*

in water, and a pH range of 8.0 to 8.5 found optimum for the bacterium's survival in the environment (**Islam et al., 1994**). Increased water temperatures can also influences *V. cholerae* abundance and during summer months, the bacterial population can increase facilitating frequent isolation of culturable *V. cholerae* O1 in environment samples (**Louis *et al.*, 2003**). Miller *et al.* (**1984**) suggested that *V. cholerae* could survive in the culturable state under low-salinity conditions (0.05%). Survival of *V. cholerae* is not related to serogroup, source (clinical or environmental), or geographical origin of the strain (**Miller *et al.*, 1984**).

The present study was conducted to know *in situ* physicochemical condition of different aquatic habitat, isolation of Vibrios, abundance of *V. cholerae* O1 in various samples and its connection with the physicochemical parameters essential for ecology of Vibrios in Bangladesh.

MATERIAL AND METHODS

Sampling sites and period

To study abundance of *Vibrio* sp. and physicochemical parameters in the fresh water body either free-living or in associated with different phytoplankton and zooplankton two month sampling was conducted in the rainy season during June and July' 2007. We collected from the pond and river at Matlab, a hyper-endemic area of cholera, about 45 km away from the southeast of Dhaka metropolis, Bangladesh (Fig.1). The latitude and longitude of this area is around 20º43′20′′ N and 90º26′10′′ E, respectively. We did five sampling each of water, phytoplankton and zooplankton sampling in every 15 d.

Sample collection and processing

We collected the subsurface water of pond and river from 0.5 to 1.0 m depth with a bucket and passed through zooplankton net (64-µm mesh size) and phytoplankton net (20-µm mesh size). The zooplankton and phytoplankton free water samples collected in sterile container. All the samples were transported to the laboratory inside a cooler box and processed in the Environmental Microbiology Laboratory of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) within 24 h of collection.
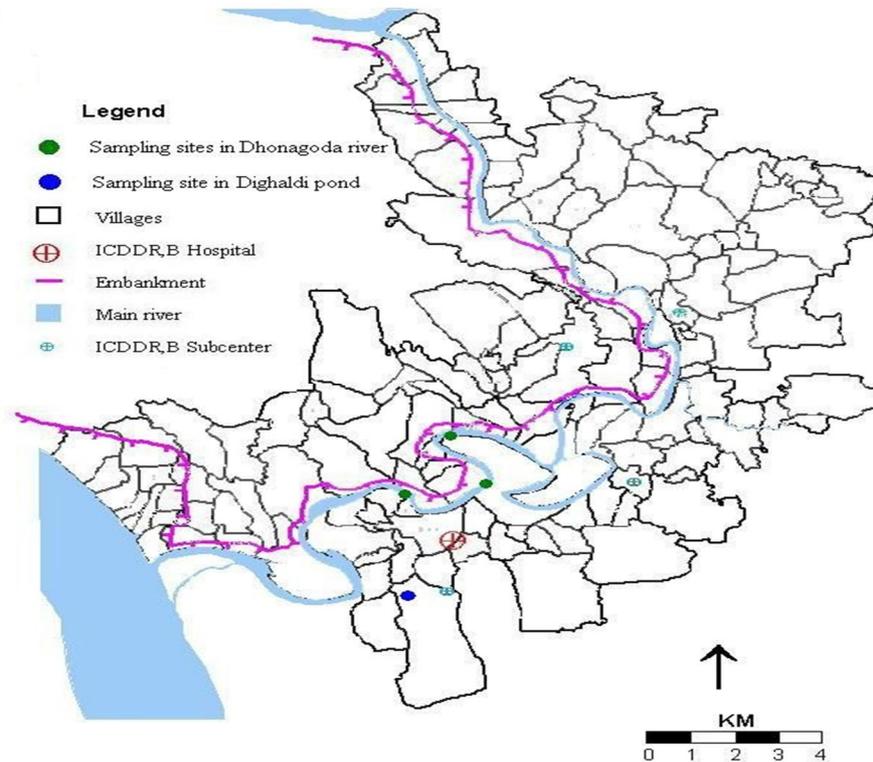
Figure 1 The map of Chandpur district showing the Sampling area

To make the zooplankton samples, phytoplankton free zooplanktons washed twice thoroughly with sterile PBS and examined the presence of phytoplankton. Phytoplankton free zooplankton samples centrifuged and crushed with sterile glass hand crusher and made 5 mL suspension with sterile phosphate buffered saline (PBS). Two mL of phytoplankton samples were also separated from zooplankton samples with sterile Pasteur pipette and homogenized using a stedfast stirrer (Model 300, Fisher Scientific, USA) and one mL of homogenate was enriched in 10 ml 1% APW (Alkaline Peptone Water; pH 9.0) and incubated 4-6 h at 37 ºC. Water, phytoplankton and zooplankton samples (450μL) enriched with 6.25-μL yeast extract and 5-μL nalidixic acid and incubated at room temperature overnight in the dark. The samples were fixed with formalin (**Kogure *et al.*, 1979**). A flow diagram of sample processing shows in figure 2.

Microbiological analyzes of samples

Hundred or ten ml processed water for microbial water analysis was passed through 0.22 μm pore size membrane filter and the filter was then placed on mFC agar plate and incubated at 37 °C and 44 °C for 18 – 20 h for counting total coliform and faecal coliform respectively. For heterotrophic plate counts, microbiological analyses were serially diluted

(10 fold dilution methods) with sterile PBS inside laminar airflow and 100 µL from each dilution were spreaded on a plate count agar (PCA) plate and incubated at 37 °C for 18-20 h.
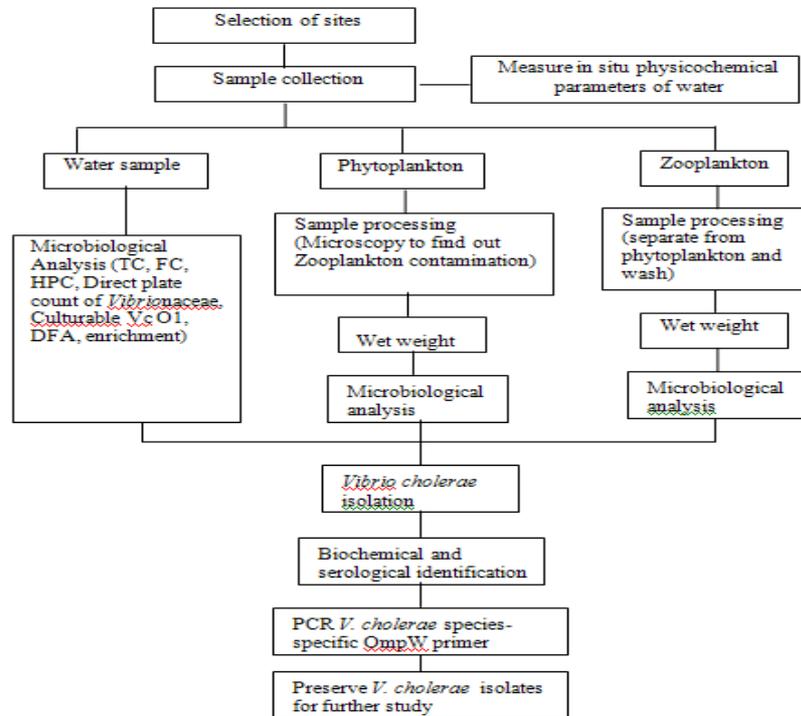
Figure 2 Diagram of surface water sampling and processing of Matlab, Bangladesh

Enumeration of total *Vibrio* sp. and identification of *V. Cholerae*

Enrichment for *Vibrio* sp. and viable bacterial counting were performed by procedure describes elsewhere (**Islam *et al.*, 2007**). In brief, One ml of homogenate enriched in alkaline peptone water (APW) and incubated for 6 h at 37 ^{o}C. Direct samples spreaded directly on dried TCBS and TTGA agar plate and incubated at 37 ^{o}C for 18-20 h and typical *Vibrio* like counted as total vibrios TCBS and black centered colonies on TTGA. From the enrichment samples in APW, 2 loopful were taken and inoculated onto TCBS and TTGA plates and incubated at 37 ^{o}C for 18-24 h. Suspected Vibrios colonies were further characterized following the procedures described earlier (**Islam *et al.*, 1995**). In brief, strains identified as *V. cholerae* if they fulfilled the following criteria: Gram negative, oxidase positive, produced acid from sucrose but not inositol and decarboxylated lysine and ornithine but not arginine. All the Strains serotyped and biotyped following the procedures described by Kelly *et al.* (**1992**).

Direct Fluorescent Antibody (DFA) Direct Viable Count

A 5 µL portion of water, phytoplankton and zooplankton samples previously processed for DFA was placed on DFA slide and the techniques was carried out following described (**Hasan *et al*., 1994, Islam *et al*., 2007**).

DNA extraction

We extracted DNA from overnight grown cultures of *V. cholereae* described protocol by Murray and Thompson (**1980**) with some modification. Briefly, overnight cultures centrifuged, and suspended in TE buffer (10mM Tris- HCl; 1M EDTA; pH-8.0) treated with 10% SDS and freshly prepared proteinase K and incubated at 50 ^{o}C for 1 h. After treatment with lysozyme, proteinase K, and ammonium dodecyl sulfate, DNA was extracted with cetyltrimethylammonium bromide and phenol-chloroform-isoamyl alcohol, concentrated with isopropanol, and resuspended in TE buffer.

Polymerase Chain reaction

DNA extracted from water and plankton samples was used as the template to detect *V. cholerae*. Simplex PCR were performed for the confirmation of outer membrane protein gene (*omp*W gene)   gene according to Nandi *et al*. (**2000**). The primers for *omp*W gene, *omp*W1 (5′-CACCAAGAAGGTGACTTTATTGTG-3′) and *omp*W2 (5′-GAACTTATAACCACCCG CG-3′). Each of the reaction mixtures contained 2.5 µL of 10X PCR amplification buffer (Invitrogen, USA), 1.0 µL of MgCl$_2$ (50 mM), 1 µL each of 10 mM dNTP (Invitrogen, USA), 1.5 µL each of the forward and reverse primers for *omp*W, 0.25µL of Taq DNA polymerase at 5 U/µL (Invitrogen, USA), and Milli-Q water to a final volume of 23.5 µL, and 1.5 µL bacterial template DNA. PCR condition was as follows: an initial denaturation at 94 °C for 5 min, followed by 35 cycles consisting of 94 °C for 1 min (denaturation), 64°C for 1 min (primer annealing), and 72 °C for 1 min (chain extension), and a final extension at 72 °C for 10 min.

Measurement of chemical parameters

The temperature, total dissolved solids (TDS), dissolved oxygen (DO) and pH were measured using portable meters (HACH Conductivity Meter, Cat. No. 51800–18; HACH Portable Dissolved Oxygen Meter, Cat. No. 51850-18; Sension TM6, CO, USA and Orion Portable pH Meter, Cat. No. 210 A; Orion Research, MA, USA).

Statistical analysis

Pearson correlation analysis ($P<0.05$ = * and $P<0.01$ = **) was carried out with the statistical SPSS (version 15) programme for Windows to examine the relation between total cultural Vibrios from water, phytoplankton, zooplankton, physicochemical parameters of water, and total phytoplankton counts from water (**SPSS, 2007**).

RESULTS

Physicochemical parameters variables and fecal contamination

Table 1 demonstrates those physicochemical parameters of water from river and pond sites and its relationship to water, phytoplankton and zooplankton by Vibrios. We found that water temperature fluctuated between 28.7^0C and 34.8^0C, pH between 6.48 and 8.88, total dissolved solids between 23.8 and 78. Total heterotropic counts between 475000 CFU/mL and 8120 CFU/mL, total coliform between 49 and 322 CFU/mL, faecal coliform between 43 and 189 CFU/mL were also reported (Table-1).

Table 1 Physiocochemical parameters of Meghna Donagoda river and adjacent pond of Matlab, Bangladesh

Sampling site	Sampling date	pH	Temp	DO	TDS	TC	FC	HPC
Pond	04.06.2007	7.88	30.8	5.28	42	140	124	8670
Pond	18.06.2007	7.39	33.3	5.35	78	63	29	57100
Pond	02.07.2007	7.8	28.7	5.12	63.8	117	87	106000
Pond	17.07.2007	6.81	32.9	6.81	62.8	56	41	14300
Pond	30.07.2007	8.88	31.5	10.34	55.7	546	472	475000
River	04.06.2007	6.95	31.9	5.84	42.5	66	58	8120
River	18.06.2007	6.71	34.8	6.41	35.7	49	43	53300
River	02.07.2007	6.48	30.2	8.21	24	69	57	185100
River	17.07.2007	7.46	31.6	6.02	28	60	45	84900
River	30.07.2007	7.18	28.7	5.27	23.8	322	189	99100

Legend: DO: Dissolved Oxygen, TDS: total dissolved solids, TC= Total coliform counts, FC=Faecal coliform counts, HPC= Heterotrophic plate count

Abundance of culturable *Vibrio* sp. in river and pond samples

Table 2 shows the abundance of *Vibrio* sp. in water, phytoplankton and zooplankton samples. Higher counts were observed in phytoplankton than zooplankton and water on both TCBS and TTGA plates. Total Vibrios were present the entire rounds counting on TCBS plates directly and counts fluctuated between log 2.77 CFU/mL and log 3.2 CFU/mL in water, in phytoplankton log 3.39 CFU/g and log 5.98 CFU/g, in zooplankton counts were between log 2.96 CFU /g and log 4.35 CFU/g (Table 2).

Enumeration of *V. cholerae* O1 by DFA technique

V. cholerae O1 was present every round except second and third round (Table 2). The DFA counts of *V. cholerae* O1 varied from 2.3 to 3.41 log cells/mL and log 3.2 to 4.24 cells/g in water and phytoplankton respectively. The *V. cholerae* O1 showed with phytoplankton in the figure 3. The zooplankton contained very few VBNC *V. cholerae* O1 (Table 2).

Table 2 Counts of *Vibrio* sp. and DFA counts of *V. cholerae* O1 at Matlab,Bangladesh

Sampling site	Sampling date	*Vibrio* sp. Counts on TCBS			*Vibrio* sp. Counts on TTGA			DFA counts of *V. cholerae* O1		
		Water	**PP***	**ZP****	**Water**	**PP**	**ZP**	**Water**	**PP**	**ZP**
Pond	04.06.2007	2.875	5.457	3.623	4.190	6.589	6.196	2.90	3.874	0
Pond	18.06.2007	3.0178	4.369	3.560	3.7481	5.7992	4.848	0	0	0
Pond	02.07.2007	3.1105	4.843	3.229	3.1105	6.3159	5.648	0	0	0
Pond	17.07.2007	2.7693	5.989	3.396	4.2648	7.9991	4.998	2.4149	4.099	2.84
Pond	30.07.2007	2.7781	4.898	4.351	3.8573	6.6872	5.918	3.3424	3.960	0
River	04.06.2007	2.8129	4.941	3.057	3.1139	7.1378	5.642	2.7781	3.796	0
River	18.06.2007	2.6812	3.426	2.985	2.6020	4.6866	5.470	2.3010	3.206	0
River	02.07.2007	2.8129	3.395	2.969	2.9637	6.0402	5.536	2.6627	3.931	2.38
River	17.07.2007	3.2174	4.648	3.019	3.5854	5.2289	5.198	2.9030	4.245	0
River	30.07.2007	3.0934	4.500	3.426	3.5563	5.9357	4.411	3.4149	4.102	0

Legend: * PP=Phytoplankton **ZP=Zooplankton

Abundance of *V. Cholerae*

Using the standard biochemical test procedure 30 isolates were identified as *V cholerae* (Table 3). Among 30 isolates, only five were found to agglutinate with *V. cholerae* O1 specific polyclonal antisera and none was positive for *V. cholerae* O139 antisera. Therefore, those five isolates were considered as *V. cholerae* O1 and others as *V. cholerae* non-O1/non-O139. Among those five *V. cholerae* O1 strains, four were isolated from pond in association with phytoplankton during the month of July when Bangladesh faced rainy season and occasionally flooding. Those five isolates were further analyzed with *V. cholerae* O1 Ogawa and Inaba specific monoclonal antisera. Two of them that were isolated from pond phytoplankton in were observed to agglutinate with Ogawa specific antisera.
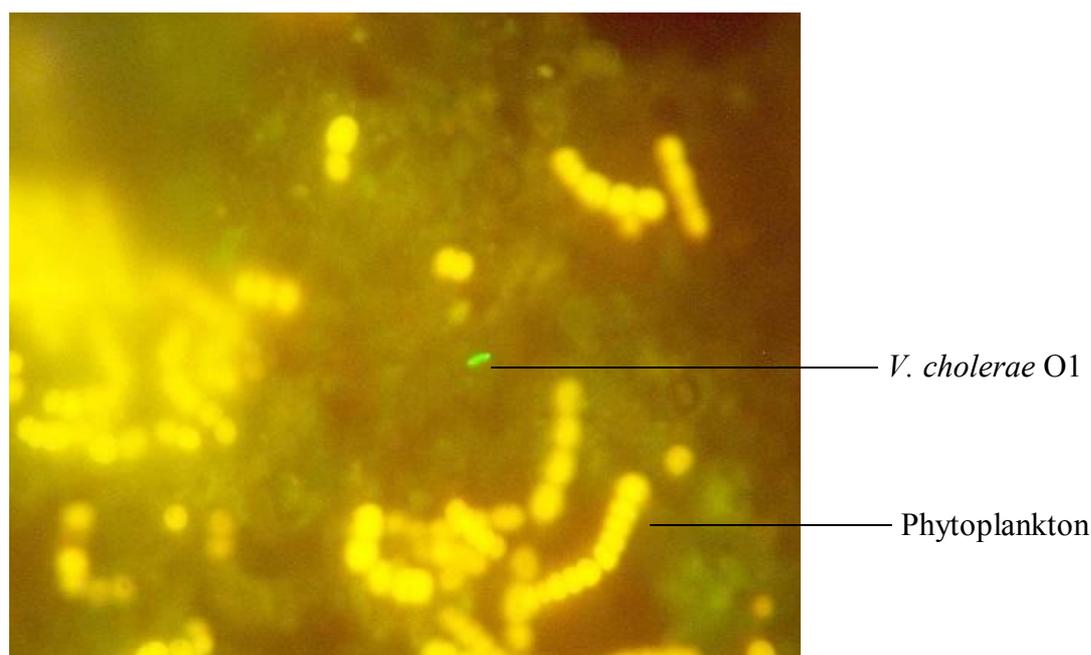
Figure 3 Detection of *V. cholerae* O1 from phytoplankton samples, Matlab, Bangladesh

Table 3 Abundance of Culturable *V. cholerae* in river and pond at Matlab, Bangladesh

Sampling site	Sampling date	*V. cholerae* non-O1/non-O139			V. cholerae O1		
		Water	PP*	ZP**	Water	PP	ZP
Pond	04.06.2007	01	01	0	0	0	0
Pond	18.06.2007	01	02	0	0	0	0
Pond	02.07.2007	01	01	0	0	0	0
Pond	17.07.2007	01	02	0	01 (Ogawa)	01 (Ogawa)	0
Pond	30.07.2007	01	01	01	01 (Inaba)	01 (Inaba)	0
River	04.06.2007	01	01	01	0	0	0
River	18.06.2007	01	01	0	0	0	0
River	02.07.2007	01	01	0	0	0	0
River	17.07.2007	01	01	0	0	0	0
River	30.07.2007	01	01	01	0	01 (Inaba)	0

Legend: * PP=Phytoplankton **ZP=Zooplankton

Detection of *omp*W gene by PCR technique

The PCR results showed that all the *V. cholerae* O1 and *V. cholerae* non-O1/non-O139 isolates carried the *omp*W gene except one strains (strain -12) (Fig. 4).
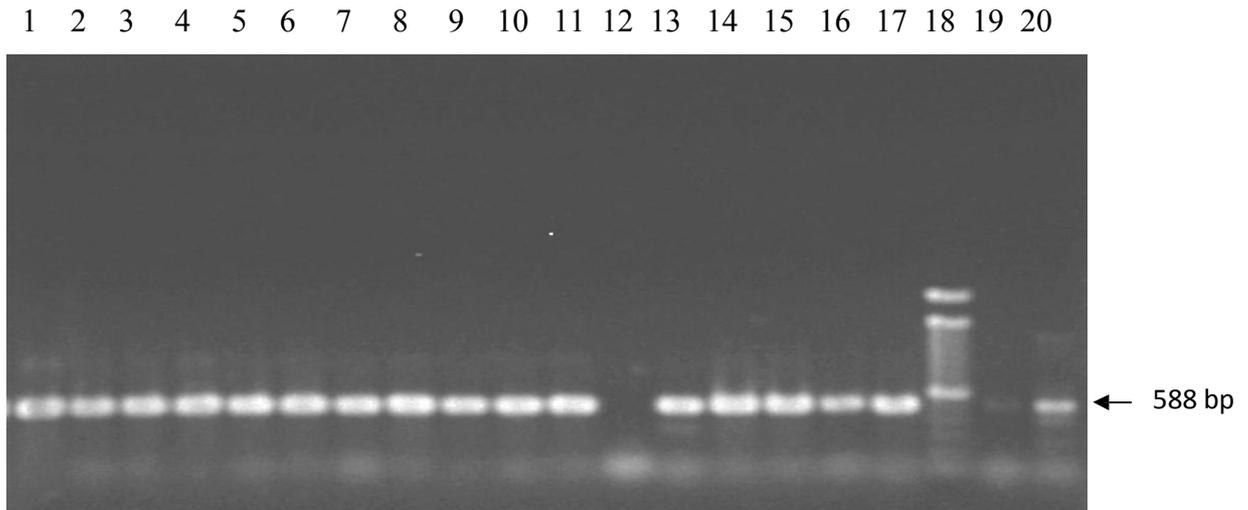
Figure 4 Agarose gel electrophoresis of PCR specific amplicon of *omp*W of *V. cholerea* isolated from different samples. Lane 1-17: *V. cholerae* isolates Lane 18: 100bp marker, Lane 19: negative control (*E. coli* ATCC13706), Lane 20: + ve control (*V. cholerea* O1 N16961).

Pearson correlation

Analysis of the data from both sites with correlation coefficient (r) revealed that total culturable Vibrios on TCBS plates was positively correlated with DO (r=0.45), phytoplankton *Vibrio* sp. (r = 0.415), zooplankton *Vibrio* sp. (r = 0.697) and negatively correlated with water temperature (r=- 0.342). A significant correlation was observed between pH (0.853*), total coliform (0.744*), faecal coliform (0.687*), heterotropic plate count (0.672*) and total Vibrios counts on water TCBS plate counts whereas water temperature and dissolved oxygen were not strongly correlated. We also observed the relationship of total *Vibrio* sp. counts in phytoplankton with all the physiochemical parameters. It was found that total *Vibrio* sp., of phytoplankton was positively correlated with pH (r=0.611), DO(r=0.170), TDS (r=0.456), total coliform (r=0.423) and faecal coliform (r=0.435) and negatively correlated with water temperature (r=-.0988) and HPC (r=-0.243). Pearson statistical analysis did not reveal any significant correlation with *Vibrio* sp. in phytoplankton on TCBS plates and physicochemical parameters. A significant correlation also found in pH (r=0.831*) and total coliform (r=0.778*) with the counts of zooplankton *Vibrio* sp. and other parameters did not showed any significant correlation. The DFA counts of water and phytoplankton revealed significant negative correlation with TDS (r=-0.715*). The positive correlation were between DFA counts of water and pH (r=0.012), DO (r=0.417), TC (r=0.357) and FC (r=0.472). Like DFA

of water, DFA counts of phytoplankton showed same correlation with TDS (r=-0.701*) and other parameters.

DISCUSSION

Vibrios are free-living in surfaces of freshwater, oceanic and estuarine environments. In aquatic environment, it may persist in associations with chitinous exoskeleton of aquatic crustacean's copepods (**Huq *et al*., 1983**), crabs, prawns, shrimps and lobsters, mucilaginous sheath of algae (**Islam *et al*., 1994**) and planktonic biofilm communities (**Islam *et al*., 2007**). The relationship of physicochemical parameters with *Vibrio* sp. population were showed in the several studies (**Costa *et al*., 2010, Patra *et al*., 2009, Shar *et al*., 2010, Sharma *et al*., 2007**).The studies indicated that total Vibrios counts is strongly correlated with water temperature, coliform, and total bacterial counts (**Sharma *et al*., 2007**) which is also agreed with the our study. We found significant correlation with total coliform, faecal coliform and total bacterial counts. Patra *et al*. (**2009**) observed the significant correlation of total *Vibrio* sp. and total coliform and faecal coliform and present study indicated the same results. The pH of water was also correlated with total Vibrios counts from water and zooplankton reported elsewhere (**Costa *et al*., 2010**). Conducting any environmental study with plankton two types of net were used, one with 64-µm mesh size and other 20-µm mesh size and usually termed zooplankton and phytoplankton net respectively (**Alam *et al*., 2006, Islam *et al*., 1994**). However, the reality was that the 20-µm mesh net sieved samples often contain few to lot of nauplii and eggs of zooplankton (**Alam *et al*., 2006**). On the other hand 64-µm mesh net sieved sample contain many large phytoplanktons such as *Volvox*, *Microcystis*, *Oscilatoria*, *Melocera* directly observed under microscope. Therefore, doing experiments with such samples might lead to bias and wrong conclusion. Under such circumstances to obtain specifically phytoplankton sample free of zooplankton and vice versa, extensive processing of sample was done. Thus, the microbiological results in association with specific plankton sample were obtained without biasness. *Vibrionaceae* abundance was found high in last two sampling in association with phytoplankton. Therefore, phytoplankton could be a better niche for *Vibrionaceae*. Islam *et al*. (**1994**) reported similar observation. Association of Vibrios with phytoplankton could be presence of mucinase in Vibrios, better nutrient accessibility in association with phytoplankton, high abundance of phytoplankton in fresh water habitat (**Islam *et al*., 1994, Islam *et al*., 2002, Martin and Bianchi, 1980, Paerl, 1978**). Other possible reason could be the stagnant nature of phytoplankton that might act as better surface

for bacterial attachment by certain chemotaxis. In water samples, faecal contamination could be a reason for high abundance of *Vibrionaceae* in water body during the last two samplings in both running water and stagnant one. On the year 2007, a severe flood happened from July to August, broke down most of the sanitation system in rural area of Bangladesh. Appearance of flood correlated with the incline of FC and TC count of river and pond. For pond, there could be another explanation as an algal bloom occurred at June that created difficulty in collection of zooplankton sample and ends during the first week of July. Algal bloom disappeared and might liberate enormous number of Vibrios as they were hypothesized as a long-term reservoir of *V. cholerae* and other Vibrios (**Islam *et al*., 1994**). Direct fluorescent antibody (DFA) count of *V. cholerae* O1 revealed their presence in both water sample and phytoplankton sample and high abundance in phytoplankton sample. Previously, Islam *et al*. (**1994**) noted the same results. *V. cholerae* O1 abundance also related to the abundance of *Vibrionaceae* in water and phytoplankton samples. Colwell *et al*. (**1981**) and Khan *et al*. (**1984**) made similar observation. The DFA counts of water, phytoplankton showed strong positive correlation with TDS but not with others physicochemical parameters and the DFA counts did not correlate with any environmental parameters (**Aulet *et al*., 2007, Binsztein *et al*., 2004**).

CONCLUSION

The result of the present study clearly concluded that *V. cholerae* present at Matlab surface water and phytoplankton and physicochemical parameters influenced the presence and survival of total Vibrios. The modified sampling processing proved *V. cholerae* associated with majority in phytoplankton for survival, and causes the epidemic cholera of this region.

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