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ATTACHMENT OF *VIBRIO CHOLERAE* SEROTYPES O1 AND O139 WITH PHYTOPLANKTON

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ABSTRACT

A total of 18 phytoplankton species were found to be associated with VCO1 in the present study. The average highest association was found to be with Chlamydomonas (49.7%) and the lowest with Cladophora (0.3%). Individual highest count of attachment was detected with Chlamydomonus in the summer of each year i.e. 53.3 %, 60.7 % and 59.9 % in the first, second and third year respectively. The Spirogyra (31.6%) and Zygnema (30.6%) had also significant attachment rate with VCO1 in the present study, whereas, the Volvox (0.6%), Oscillatoria (0.4%), Pithophora (1.1%) and Oedogonium (1.1%) had a very lower proportions of attachment with the same bacteria. The average highest and lowest count of attachment of phytoplankton with V. cholerae O139 was found to be with Chlamydomonas (10.3%) and Cladophora (0.78%) respectively. However, all phytoplankton had very meager proportions of attachment with V. cholerae O139 than that of their attachment with V. cholerae O1. The highest count of phytoplankton was found to be between February to April (post winter or autumn) and August to September of each studied year throughout the study period and the lowest count of different phytoplanktons was found in winter months and summer months. Such findings of phytoplankton bloom are directly linked with two cholera peaks (autumn and spring) in Bangladesh.

Keywords: *Chlamydomonus*, *Cladophora*, Phytoplankton, *Spirogyra*, *V. cholera*

Introduction

Cholera is a major public-health problem in developing countries, caused by infection of the intestine with toxigenic *Vibrio cholerae* (Islam *et. al*., 1995). For a long time, it was not understood that the epidemic strain of *V. cholerae* was a bacterium naturally occurring in the aquatic environment (Colwell, 1996). It is now recognized that *V. cholerae* is a component of coastal and estuarine microbial ecosystems, with the copepod species of zooplankton that comprise the aquatic fauna of rivers, bays, estuaries and the open ocean serving as host for the bacterium (Colwell *et. al.,* 1977; Huq *et. al.,* 1984; Islam *et. al.,* 1995; Kaper *et. al.,* 1992). *V. cholerae* can be found to be attached to the carapace and in the gut of copepods in large numbers, the copepod essentially serving as a vector for this human pathogen (Colwell, 1996; Nalin *et. al.,* 1979).

Cholera is endemic in Bangladesh and maintains a regular seasonal pattern (Glass *et. al.,* 1982). In Bangladesh, cholera epidemics occur twice every year, the highest peak during post monsoon (September-January) and second smaller peak during pre monsoon (March-May). During inter-epidemic period *V. cholerae* cannot be cultured from the surface water, whereas in epidemic season it can be isolated from the patients' body as well as from surface water (Alam *et. al.,* 2006).

V. cholerae O1 is native to both marine and freshwater environments where it exists in association with planktons (Albert *et. al.,* 1993). In general, it can be isolated from only 1% of water samples collected during epidemic periods and rarely, if ever, between epidemics (Almeida *et. al.,* 1990). However, fluorescent antibody-based studies show that *V. cholerae* O1 is nevertheless, present in aquatic environments throughout the year (Baumann *et. al.,* 1984). Evidences show that *V. cholerae* O1 becomes coccoid and enters into a non-culturable state in the environment when conditions are not conductive to active growth. Some of the coccoid non-culturable cells can retain their metabolic activity for a prolonged time (Baine *et. al.,* 1974). During epidemic period, environmental stress situations in aquatic environments such as low concentration which allows *Vibrio cholerae* to maintain a metabolic functions but it cannot be cultured *in vitro*. If conditions become favourable again it can revert to the culturable state (Xu

et. al., 1982; Rollins and Colwell, 1986; Roszak and Colwell, 1987; Nilsson *et. al.*, 1991; Borroto, 1997 and Louis *et. al.*, 2003).

Field studies in the Bay of Bengal have not been done, hence, little is known about the geographic distribution of toxigenic strains of *V. cholerae* O1 and O139 (Alam *et. al.*, 2006). Study on association of *V. cholerae* with phytoplankton is relatively less.

Considering all, the current study was designed to add some light to the debate regarding the ecology of *Vibrio cholerae*, a pressing global concern as cholera is becoming pandemic day by day, possible correlation between survival of *Vibrio cholerae* and their nature of biological attachment with ramified planktons especially phytoplanktons. These information will draw up a road map for future research in multidimensional areas for the greater interest of public health across the globe and the findings may help to devise ways to combat cholera epidemics and to curb its grave threat to health for the sake of peaceful and productive living.

Materials and Methods

The present study was conducted at three selected ponds of Mathbaria, which is geographically adjacent to the coast of the Bay of Bengal and approximately 400 km southwest of Dhaka. The geographical location of the study area was between 22° 29' N to 90°-22' E. Several coastal ponds of Mathbaria were surveyed by the author along with a team from Enteric Microbiology Laboratory of Laboratory Science Division (LSD) under International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) and finally three pristine ponds were chosen. The chosen ponds were considered as: pond 1(site-1), pond 2 (site-2) and pond 3 (site-3) and those ponds are learnt to be the potential reservoir of *Vibrio cholerae* round the year. The selected ponds were also given credits among other ponds as they retain water throughout the year and are not contaminated by effluent from outside sources.

A total of 108 phytoplankton samples were analyzed for consecutive 36 months (3 years). In each round, one 5 liter sampling bottle was filled with water for 20 times from different areas of each pond and the same were filtered through 20-µm pore-sized nylon nets (Millipore Corp., Bedford, and Mass). In this way, 100 liters of water was filtered from each pond in each round in order to get the final concentration of 50 ml with a view to analyzing zooplanktons along with their possible attachment with *Vibrio cholerae*. From each of 50 ml samples, 10 ml was

transferred into another small vial along with preservative (formalin for zooplankton). The same trend of sample collection was continued for consecutive 36 months totaling the number of samples as 108. All samples were collected by using aseptic technique in sterile dark Nalgene bottles (Nalgene Nunc International, St. Louis, Mo.) and transported at ambient air temperature from the site of collection to the central laboratory of the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), in Dhaka. The samples from 20-µm mesh sized plankton nets were further concentrated in the laboratory to a final volume of 5 ml by filtering through a 0.22-µm-pore-size bacteriological membrane filter (Millipore) and the retained contents on the membrane filter were washed into phosphate-buffered saline (pH 8.0). All samples were processed the following day, with approximately 20 hours of elapsing between sample collections in the field and processing in the laboratory.

Sampling Technique

A total of 108 samples comprising of phytoplankton samples were analysed for consecutive 36 months (3 years). In each round, one 5 liter sampling bottle was filled with water for 20 times from different areas of each pond and the same were filtered through 20-µm-pore-sized nylon nets (Millipore Corp., Bedford, and Mass). In this way, 100 liters of water was filtered from each pond in each round in order to get the final concentration of 50 ml with a view to analyzing phytoplankton along with their possible attachment with *Vibrio cholerae*. From each of 50 ml samples, 10 ml was transferred into another small vial along with preservative (Lugol's iodine). The same trend of sample collection was continued for consecutive 36 months. All samples were collected by using aseptic technique in sterile dark Nalgene bottles (Nalgene Nunc International, St. Louis, Mo.) along with preservative placed in an insulated plastic box, and transported at ambient air temperature from the site of collection to the central laboratory of the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), in Dhaka. The samples from 20-µm mesh sized plankton nets were further concentrated in the laboratory to a final volume of 5 ml by filtering through a 0.22-µm-pore-size bacteriological membrane filter (Millipore) and the retained contents on the membrane filter were washed into phosphate-buffered saline (pH 8.0). All limnological parameters like pH, dissolved oxygen (DO), temperature of water and

Analysis of phytoplankton

From 50 ml, 10 ml was for analysis and the samples were immediately preserved by 5% buffered formaldehyde. For qualitative and quantitative study, samples were observed under a compound microscope in a S-R (Sedgwick-Rafter) cell. Sedgwick- Rafter (S-R) counting cell is a device commonly used for counting plankton. Before filling the S-R cell with sample the cover slip was placed diagonally across the cell. The sample was transferred with a large pipette. Placing the cover slip in this manner is to prevent formation of air bubbles. Then cover slip was rotated to cover the inner portion and then count was made under microscope.

Let, 1 ml conc. sample contains nCFU

So, 5 ml conc. sample contains 5 nCFU

As, count of 40 ml sample = count of conc. 5 ml

So, 40 ml sample also contains 5n CFU

50 ml sample will contain = (5n x 50) / 40 CFU = 6.25 x nCFU

Again, CFU count of original 100 L sample = CFU count of conc. 50 ml

So, 100 L (10^5 ml) water sample also contains = 6.25 x nCFU

So, 1 ml of water sample will contain = (6.25 x n) / 10^5 CFU= 6.25 x 10^5 x n CFU

CFU count of 1 ml water sample = 6.25 x 10^{-5} x CFU of 1 ml concentrated sample

DFA = 1.25 x 10^1/L = (6.25 x 10^{-5} x 200 x 1000).

(No. of bacterial colonies were multiplied by the conservation factor)

Samples were enriched in alkaline peptone water referred as APW (Difco, Detroit, MI) and incubated at 37°C for 6 to 8 hours before plating on TCBS agar (Eiken, Tokyo, Japan) and TTGA (Difco). APW contains 1% peptone and 1% sodium chloride with the pH adjusted to 8.5. Approximately, 5 µL of enriched APW broth was streaked by using an inoculating loop on both thiosulfate-citrate-bile salts-sucrose (TCBS), and taurocholate-tellurite-gelatin agar (TTGA) and incubated at 37°C for 18 to 24 hours. TCBS and TTGA are two of the most commonly used and most widely studied selective plating media for cholera pathogen. colonies with the characteristic appearance of *Vibrio cholerae* were confirmed by biochemical tests like KIA (Kligler's iron agar), TSI (triple sugar iron agar,oxidase, gas production from glucose, sucrose, lysine, arginine, ornithine, VP (Voges-Proskaeur) etc. Finally, serological tests tests were done using polyvalent and monoclonal antibodies specific for *V. cholerae* O1 and O139. Samples were preincubated

overnight, in the dark, with 0.025% yeast extract (Difco) and 0.002% nalidixic acid (Sigma-Aldrich, St. Louis, MO). The samples were then centrifuged and the pellet was stained with cholera DFA reagents like fluorescein isothio cyanate-labelled antiserum specific for O1 or O139 (New Horizon Diagnostics, Columbia, MD). Fluorescent stained cells were observed and counted under UV light by using an epifluorescence microscope (Olympus Bx51) and recorded with the help of a digital camera attached with the same microscope (Olympus DP20).

Results and Discussion

Table 1. Average percentage of phytoplanktons in association with *Vibrio cholerae* O1

Site	Group	2006-2007			2007-2008			2008-2009			Average (%)
		Winter (%)	Summer (%)	Monsoon (%)	Winter (%)	Summer (%)	Monsoon (%)	Winter (%)	Summer (%)	Monsoon (%)	
O1	*Chlamydomonas.*	46.8	53.3	42.9	41.5	60.7	49.1	43.9	59.9	48.9	49.7
	Volvox	ND	ND	ND	ND	ND	ND	0.6	3.4	1.7	0.6
	Pediastrum	ND	ND	ND	6.4	26.1	12.9	8.1	23.8	17.3	10.5
	Cladophora	0	1	0.4	0	1.2	0	ND	ND	ND	0.3
	Ulothrix	ND	ND	ND	5.3	10.2	7.1	5.9	11.3	7.6	5.3
	Spirogyra	26.3	37.8	29.7	27.1	38.5	29.9	25.9	38.1	31.3	31.6
	Zygnema.	ND	ND	ND	39.9	50.5	46.1	41.4	51.1	46.5	30.6
	Oedogonium	ND	ND	ND	ND	ND	ND	2.2	4.6	3.2	1.1
	Cosmarium	ND	ND	ND	1.1	8.7	4.4	6	11	4	3.9
	Pithophora	0	7	2.9	ND	ND	ND	ND	ND	ND	1.1
	Carteria	ND	ND	ND	ND	ND	ND	2	0	1	0.33
	Batrachospermum	2.5	16.3	5.8	ND	ND	ND	ND	ND	ND	2.7
	Ceramium	9.3	17.7	11.6	ND	ND	ND	23	3	4	7.6
	Nostoc	0.1	8.5	3.6	0.1	6.9	3.1	0.2	7.6	4.9	3.9
	Anabaena	ND	ND	ND	2.4	13.2	7.6	2.1	12.9	7.1	5

Gloeotrichia	ND	ND	ND	1.7	15	10.4	1.9	14.8	10.9	6.1
Oscillatoria	ND	ND	ND	ND	ND	1	ND	1	2	0.4
Navicula	5.7	12.8	7.1	4.9	12.3	6.3	5.3	11.9	4.8	7.9

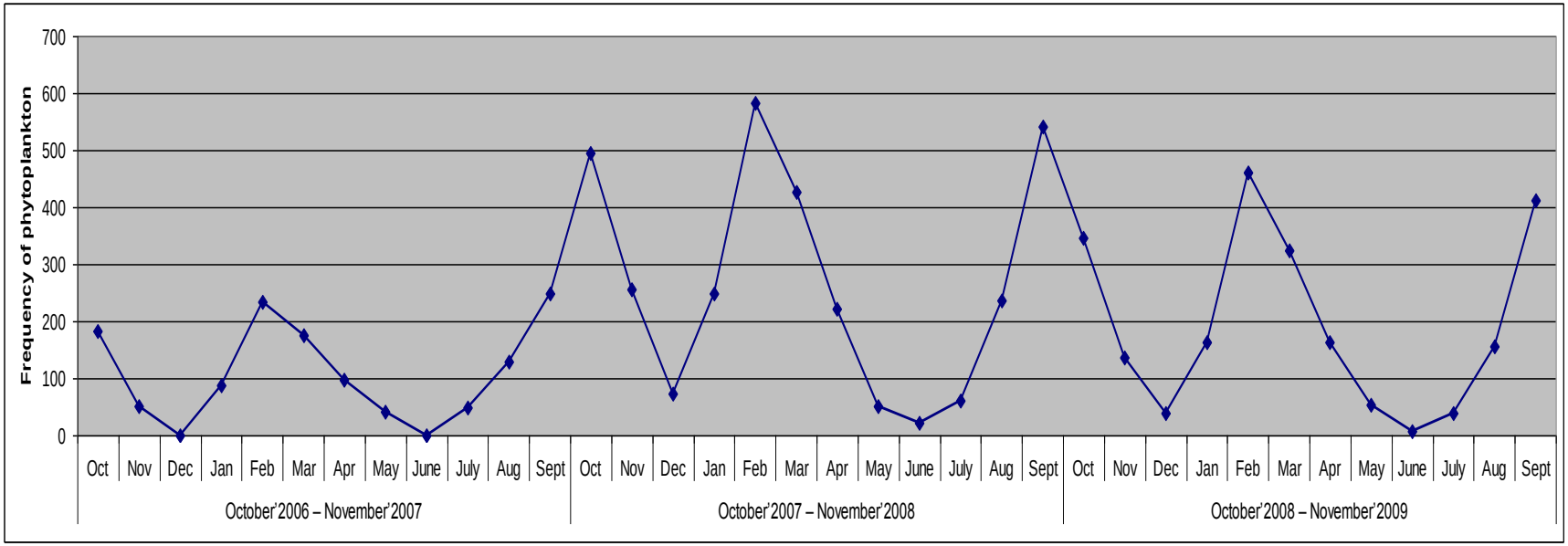
A total of 18 phytoplankton species were found to be associated with VCO1 in the present study. The average highest association was found to be with *Chlamydomonas* (49.7%) and the lowest with *Cladophora* (0.3%). Individual highest count of attachment was detected with *Chlamydomonus* in the summer of each year i.e. 53.3 %, 60.7 % and 59.9 % in the first, second and third year respectively. The *Spirogyra* (31.6%) and *Zygnema* (30.6%) had also significant attachment rate with VCO1 in the present study, whereas, the *Volvox* (0.6%), *Oscillatoria* (0.4%), *Pithophora* (1.1%) and *Oedogonium* (1.1%) had a very lower proportions of attachment with the same bacteria (Table 1).

Table 2. Average percentage of phytoplankton in association with *Vibrio cholerae* O139

Site	Group	2006-2007			2007-2008			2008-2009			Average (%)
		Winter (%)	Summer (%)	Monsoon (%)	Winter (%)	Summer (%)	Monsoon (%)	Winter (%)	Summer (%)	Monsoon (%)	
1	*Chlamydomonas.*	6.8	13.3	10.9	6.5	16.7	9.1	6.9	13.9	8.9	10.3
	Volvox	0.3	2.3	1.4	0.4	2.7	1.3	ND	ND	ND	0.93
	Pediastrum	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Cladophora	0.3	1.3	0.2	0.1	1.6	0.7	0.3	1.7	0.8	0.78
	Ulothrix.	2.6	3.7	2.9	2.7	3.8	2.9	2.5	3.8	3.1	3.11
	Pithophora	1.2	4.9	3.3	ND	ND	ND	ND	ND	ND	1.04
	Spirogyra	3.3	5.2	4.1	3.4	5.7	4.3	ND	ND	ND	2.89
	Zygnema.	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Oedogonium	0.1	2.6	1.6	0.1	2.9	1.1	0.2	2.6	1.9	1.46
	Cosmarium	2.7	4.8	3.1	2.9	4.3	3.3	2.3	4.9	3.8	3.57
	Carteria	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Batrachospermum	0.9	4.2	3.1	0.7	4.3	3.2	0.8	4.5	3.4	2.79
	Ceramium	ND	ND	ND	1.7	6.1	3.3	1.1	6.2	3.7	2.46

It is mention-worthy that *Nostoc*, *Anabaena*, *Gloeotrichia* and *Oscillatoria* have been found to have no association with *V. cholerae* O139 in the present study, although they showed their association with *V. cholerae* O139. The average highest and lowest count of attachment of phytoplankton with *V. cholerae* O139 was found to be with *Chlamydomonas* (10.3%) and *Cladophora* (0.78%) respectively. However, all phytoplankton had very meager proportions of attachment with *V. cholerae* O139 than that of their attachment with *V. cholerae* O1. It is also worth mentioning that all the individual highest counts of attachment between *Chlamydomonas* and *V. cholerae* O139 were exclusively found in the summer months of every year i.e. 13.3%, 16.7% and 13.9% in the first, second and third year respectively and the same organism had all the lowest counts of attachment (6.8%, 6.5% and 6.9% in the first, second and third year respectively) in the winter season of every year (Table 2).

Figure 1. Average frequency of phytoplankton in all sites during three studied years



The highest count of phytoplankton was found to be between February to April (post winter or autumn) and August to September of each studied year throughout the study period and the lowest count of different phytoplanktons was found in winter months and summer months. Such findings of phytoplankton bloom are directly linked with two cholera peaks (autumn and spring) in Bangladesh. Such correlation signifies the role of phytoplankton to support *V. cholerae* O1 and O139 as well as cholera epidemics. So, prior to control or eradicate cholera pathogen it deserves to control plankton. Plankton on the other hand is of tremendous importance as they provide the principal natural food to aquatic organism especially the fish. Hence, *V. cholerae* may be integral part of numerous aquatic food chains. Control of *Vibrio cholerae* should be based on biological in nature for the greater interest of ecological balance and diversity (Figure 1)

In the present study, the total counts of VCO1 in water, zooplankton and phytoplankton samples were $2.2x10^7$ cfu/L, $7.0x10^5$ cfu/L and $4.0x10^5$ cfu/L respectively and the total counts of VCO139 were $5.0x10^6$ cfu/L, $4.6x10^5$ cfu/L and $3.4x10^5$ cfu/L respectively. Unlike the findings of Ahmed *et. al.* (2007), there was the blooms of *Chlamydomonas* (1345/L) and swarm of *Asplanchna* (3519/L) immediately before the cholera epidemics (between February to March and September to October). Ahmed *et. al.* (2007) found an association of *Vibrio cholerae* with plankton especially cyanobacteria in relation to some physicochemical parameters in the river Buriganga, Dhaka, Bangladesh. Monthly abundance of phytoplankton and zooplankton varied from 457 to 14166 and from 169 to 1655 individual in L^{-1}, respectively. Monthly average of fecal coliform in water, zooplankton and phytoplankton samples were 3.99×10^9, 4.54×10^3 and 4.28×10^2 (CFU L^{-1}), respectively. During epidemics, toxigenic *V. cholerae* O1 and O139 were isolated from the surface water and were also isolated from plankton samples. It was also observed that ctx (cholera toxin) positive in water and phytoplankton samples of the river. A bloom of *Oscillatoria* sp. (1.6×10^4 individual L^{-1}) occurred in the upper reaches of the river Buriganga.

In the present study, Diatoms counts as well as its association with VCO1 and O139 was found less than that of other phytoplankton like *Chlamydomonas, Oedogonium, Zygnema* and *Oscillatoria* and highest average association of VCO1 was found to be with *Chlamydomonas* (49.7%, 68.4% and 55.9% in site 1, 2 and 3 respectively) followed by *Spirogyra* (31.6% and 58.3% in site 1 and 2 respectively and 35.8% with *Pithophora* in site 3) and *Zygnema* (30.6%, in site 1 and 46.8% in site 3). However, *Nizschia* was not at all detected in the current study. Seeligmann *et. al.* (2008) found through analysis of phytoplankton that diatoms were predominant with percentages between 85 and 100%. *Nitzschia palea* was the only species found at all three sampling sites with percentages between 0 and 38%. Of the 54 samples obtained during the 18 sampling periods, *V. cholerae* VNC was detected through direct immunofluorescence in 39% of the cases and at all three sampling sites. Positive samples were analysed for association of VNC with phytoplankton and between 1 and 10 bacteria were found adhered to a single algal cell. This confirms for the first time in northwestern Argentina adherence of this microorganism to the genera *Stigeoclonium and Nitzschia* as environmental

reservoirs. No correlation could be found between the latent form of vibrio and the environmental variables assayed.

Conclusion

The current study revealed that there is a correlation between pathogenic *Vibrio cholerae* and phytoplankton bloom. A simple and inexpensive filtration method to sieve out plankton to which *Vibrio cholerae* are attached in raw water supplies, such as ponds, rivers and other natural water supplies could be an effective way to curb or at least to reduce the number of cholera epidemics. This can be done by traditional filtration of pond or river water using cotton 'sari' worn by the women community prior to domestic use or drinking so that *Vibrio cholerae* attached with planktons can be reduced. Further studies regarding possible biological association between these non-Vibrio cholera pathogen and aquatic micro- and macro flora and fauna should be accomplished to reach a consensus regarding overall ecological niche of cholera causing pathogen so that cholera epidemics can be managed well in an integrated manner keeping in mind the interest of biodiversity and ecological balance.

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