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Association of pathogenic *Vibrio cholerae* O1 and O139 with zooplankton

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ABSTRACT

Vibrio cholerae O1 was found to be associated with six groups of zooplanktons during the study period. The study revealed that V. cholerae O1 had maximum association with rotifer (60.4%) and nauplii (76%) and minimum association with cladocera (3.39%). Vibrio cholerae O139 was also found to be associated with six groups of zooplanktons in three studied sites during the study period. The data revealed that V. cholerae O139 had highest association with rotifera in all sites (79.5%, 76% and 77.7% respectively) and lowest with copepod (2.46%) and ostracoda (2.69%). Zooplankton had more or less two peaks of swarm or bloom like from February to April (post-winter or spring) and September to November (pre-winter or autumn) which coincides with two seasonal cholera epidemics in Bangladesh. The findings therefore suggest a correlation between cholera epidemics and zooplankton bloom.

Keywords: *Vibrio cholerae* (VC), zooplankton, rotifer, cladocera, copepod.

INTRODUCTION

Cholera is a major public-health problem in developing countries, caused by infection of the intestine with toxicogenic *Vibrio cholerae* [1]. For a long time, it was not understood that the epidemic strain of *V. cholerae* was a bacterium naturally occurring in the aquatic environment [2]. 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 [3, 4, 1, 5]. *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 [2, 6]. A single copepod, for example, can contain as many as 10^3–10^5 *V. cholerae* cells [7]. Because a concentration of 10^9 ml^{-1} *V. cholerae* comprises an infective dose, ingestion of untreated water containing a relatively small number of copepods carrying *V. cholerae* can initiate the disease [8]. Therefore, conditions favorable for multiplication of copepods and related chitinous zooplankton species for which *V. cholerae* is commensal or symbiotic will result in an increase in the number of *V. cholerae*. The importance of copepods in cholera transmission was demonstrated in a study showing that the number of cholera cases in Bangladeshi villages was significantly reduced when a simple filtration method that effectively removed the plankton and particulate matter was used to treat drinking water [9, 2]. Laboratory research [10, 11] later confirmed this theory, supporting the hypothesis that the microorganism is an autochthonous member of the microbial flora found in brackish waters typical of estuaries and coastal swamps, as was first suggested by [3].

Cholera is endemic in Bangladesh and maintains a regular seasonal pattern [12]. 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 [13].

V. cholerae O1 is native to both marine and freshwater environments where it exists in association with planktons [14]. In general, it can be isolated from only 1% of water samples collected during epidemic periods and rarely, if ever, between epidemics [15]. However, fluorescent antibody-based studies show that *V. cholerae* O1 is nevertheless, present in aquatic environments throughout the year [16]. 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 [17]. 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 [18, 19, 20, 21, 22, 23].

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* versus physical and chemical parameters of water and their nature of biological attachment with ramified planktons especially zooplanktons. As cholera is one of the major threats to human health and its related problems are becoming insurmountable day by day, 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 zooplankton 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 64-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 totalling 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 64-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.

Analysis of zooplankton

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. 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 n Colony Forming Unit (CFU)

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 ١. Average percentage of zooplanktons in association with *Vibrio cholerae* O1

Sites	Group	Winter (%)	Summer (%)	Monsoon (%)	Average (%)
1	Protozoa	8.6	28.1	16	17.6
	Rotifera	48	75	58.3	60.4
	Nauplii	11	46.6	38	31.9
	copepoda	3.9	11.8	8.7	8.13
	Cladocera	3	4.87	2.3	3.39
	Ostracoda	0	0	0	0
2	Protozoa	21.9	24.8	21	22.6
	Rotifera	70.7	70.4	78	73
	Nauplii	43.9	50	45	46.3
	copepoda	4.93	8.83	7.4	7.05
	Cladocera	6.83	8.8	7	7.54
	Ostracoda	0.63	1.67	1.1	1.13
3	Protozoa	1.23	4.1	2.4	2.58
	Rotifera	46.5	55	50	50.5
	Nauplii	72.1	80	76	76
	copepoda	8.43	13.2	11	10.9
	Cladocera	0.33	4.87	1.7	2.3
	Ostracoda	14.5	20.7	17	17.4

Vibrio cholerae O1 was found to be associated with six groups of zooplanktons during study period. The study revealed that *V. cholerae* O1 had maximum association with rotifera in both site 1 (60.4%) and site 2 (73%) but in site 3, maximum association was found to be with nauplii (76%). In site 1 and 2, *V. cholerae* O1 had minimum association with cladocera (3.39%) whereas, in site 3 there was minimum association with cladocera (2.3%). No association between ostracoda and *V. cholerae* O1 was found in site 1. *Nauplii* had also significant association with *V. cholerae* O1 both in site 1 (31.9%) and site 2 (46.3%) (Table ١).

Table ٢. Average percentage of zooplanktons in association with *Vibrio cholerae* O139

Sites	Group	Winter (%)	Summer (%)	Monsoon (%)	Average (%)
1	Protozoa	2.23	8.03	2.8	4.35
	Rotifera	70.2	88.4	80	79.5
	Nauplii	21.8	36.2	26	28
	Copepoda	1.13	3.6	1.5	2.08
	Cladocera	1.43	5.73	2	3.05
	Ostracoda	0.87	7	2.2	3.36
2	Protozoa	0.3	7.5	1.8	3.2
	Rotifera	70.8	85.3	72	76
	Nauplii	22	35.8	28	28.6
	Copepoda	1.17	4.3	1.9	2.46
	Cladocera	0.73	6.6	3.6	3.64
	Ostracoda	0.77	6.17	2.7	3.21
3	Protozoa	2.47	8.07	3.7	4.75
	Rotifera	71.1	87	75	77.7
	Nauplii	23.1	35.1	29	29.1
	Copepoda	1.53	4.33	2.3	2.72
	Cladocera	1.27	4.87	2.2	2.78
	Ostracoda	0.2	5.57	2.3	2.69

Vibrio cholerae O139 were also found to be associated with six groups of zooplanktons in three studied sites during the investigation period. The data revealed that *V. cholerae* O139 had highest association with rotifera in all sites (79.5%, 76% and 77.7% in site 1, 2 and 3 respectively) and lowest with copepoda both in site 1 (2.08%) and 2 (2.46%), whereas, in site 3 it showed lowest association with ostracoda (2.69%). Nauplii also showed connection with VCO139 in all sites (28%, 28.6% and 29.1 % in site 1, 2 and 3 respectively) indicating its potential role in supporting the survival of VCO139. Rotifera showed highest attachment in summer in all individual sites (88.4%, 85.3% and 87% in site 1, 2 and 3 respectively) and lowest in monsoon (70.2, 70.8 and 71.1% in site 1, 2 and 3 respectively)(Table ٢).

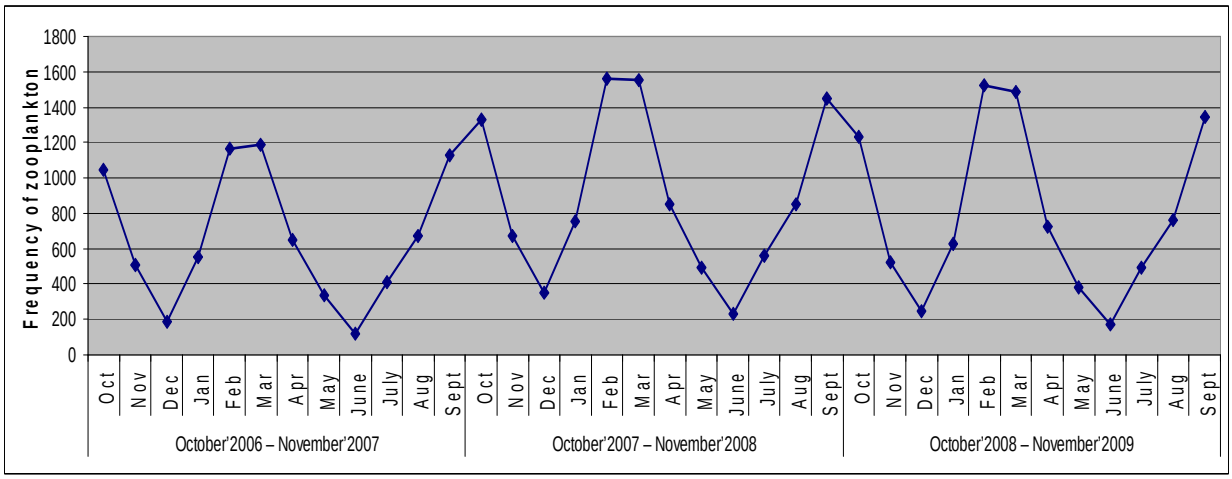

Figure ١. Average number zooplankton (per liter) from all sites during three years of study

The graphical presentation showed that zooplankton had more or less two peaks of swarm or bloom periods, from February to April (post-winter or spring) and September to November (pre-winter or autumn). These two seasons are considered as seasonal cholera epidemics in Bangladesh according to [13]. So, a correlation exists between cholera epidemic and zooplankton bloom (Figure ١).

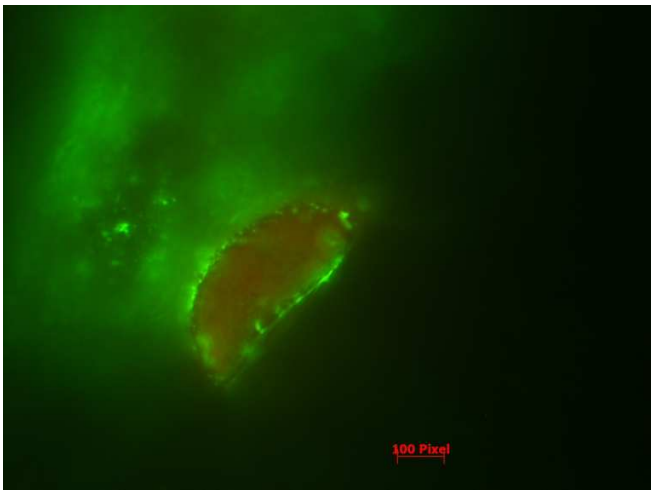

Plate ١. *Vibrio cholerae* attached with *Trichocera*

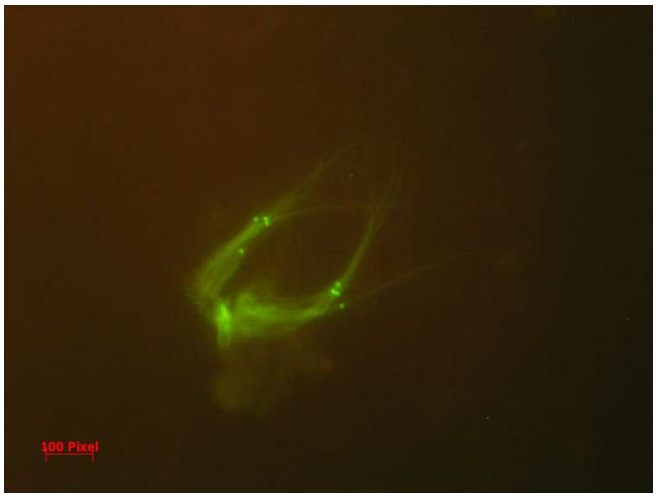

Plate ٢. *Vibrio cholerae* attached with *Nauplius*

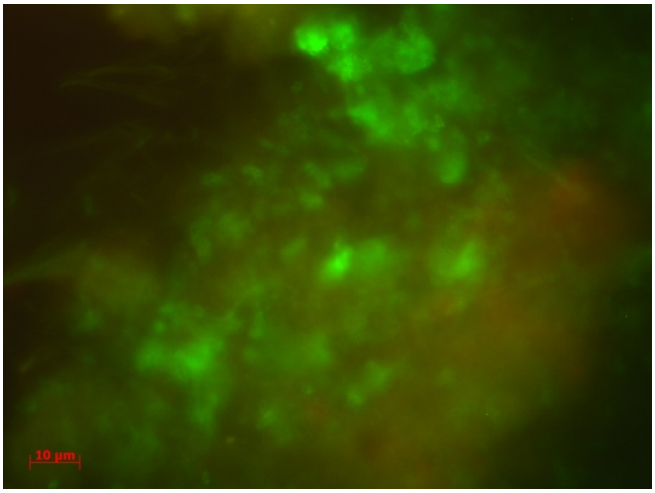

Plate ٣. *Vibrio cholerae* attached with *Lecane* (a Rotifer)

Plate ٤. *Vibrio cholerae* attached with *Brachionus*

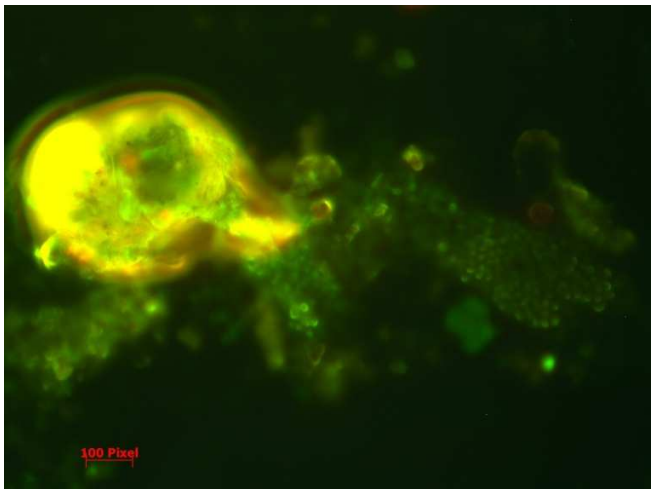

Plate ٥. *Vibrio cholerae* attached with *Asplanchna* (a Rotifer)

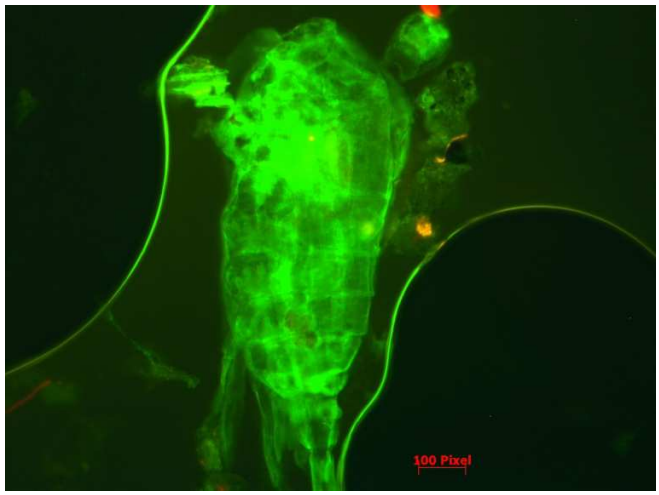

Plate ٦. *Vibrio cholerae* attached with *Cyclops* (a Copepod)

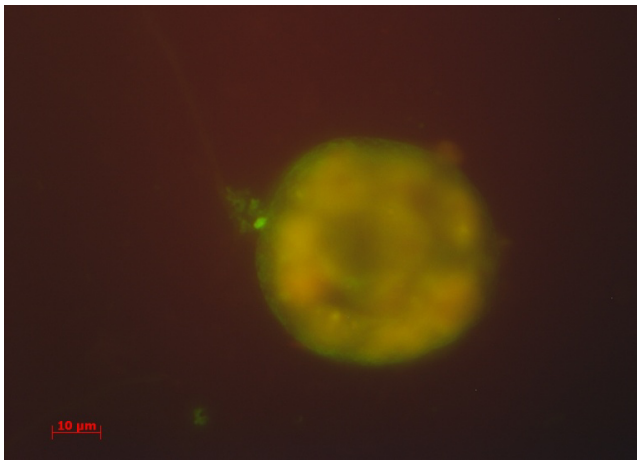

Plate ٧. *Vibrio cholerae* attached with *Polyarthra* (a Rotifer)

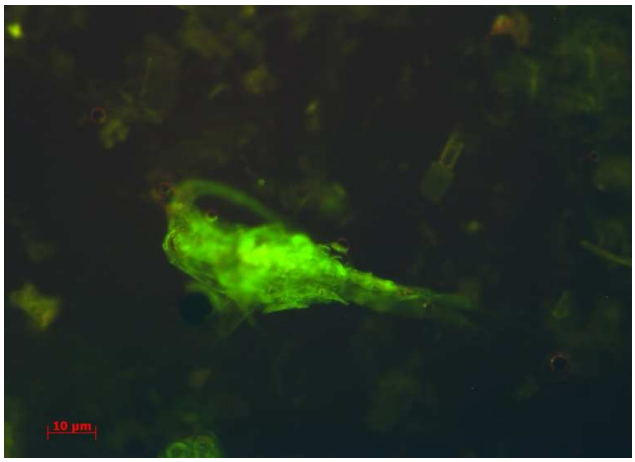

Plate ٨. *Vibrio cholerae* attached with *Nebalia* (a protozoan)

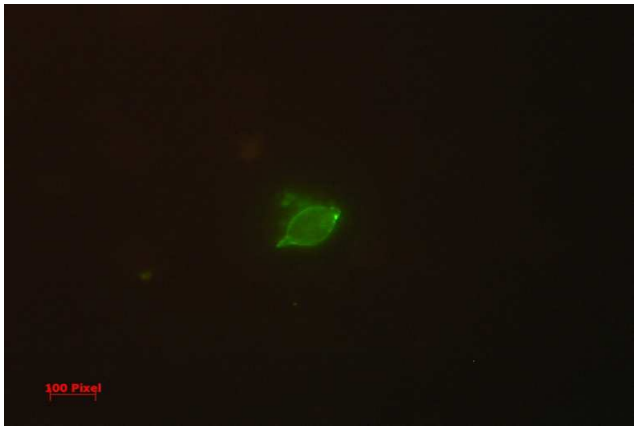

Plate ٩. *Vibrio cholerae* attached with *Daphnia* (a Copepod)

Plate ١٠. *Vibrio cholerae* attached with *Brachionus falcatus*

The present study reported association of VCO1 an O139 with diverse groups of zooplankton. Rotifera fauna under zooplankton and *Chlamydomonas* under phytoplankton showed maximum predilection for both VCO1 and O139. [24] demonstrated that the persistence of culturable *V. cholerae* O1 under laboratory conditions for over 15 months in association with blue green bacteria (cyanobacteria).

The current study also found association of VCO1 and O139 with zooplanktons under protozoa, rotifera, ostracoda, nauplii, cladocera, copepoda. [25] found that four out of five clinical *V. cholerae* O1 strains and endogenous bacterial flora were attached in approximately equal numbers to both exuviae of copepoda and whole specimens especially *V. cholerae* O1 were found to remain attached with several phytoplankton species. They also showed that *V. cholerae* O1 can bind to diverse plankton species collected from an area where cholera is an endemic disease, with potentially significant effects on its ecology.

The present study found correlation between plankton blooms and flourishing trend of VCO1 and O139. In addition to association with copepods, the current study also reported association with protozoa, rotifera, ostracoda, nauplii and cladocera. These information provides strong evidence in favour of cholera epidemics being climate-linked. [26] reported that sea surface temperature shows an annual cycle similar to the cholera case data. Sea surface height may be an indicator of incursion of plankton-laden inland water, e.g., tidal rivers, because it was also found to be correlated with cholera outbreaks. The extensive studies confirmed *V. cholerae* is autochthonous to the aquatic environment and is a commensal of zooplankton, i.e., copepods, when combined with the findings of the satellite data analyses, provide strong evidence that cholera epidemics are climate-linked. Such findings will help to control cholera epidemics in a better way.

The present study reports consolidate the findings of [13] to consider the aquatic environment of Mathbaria zone to be the potential reservoir of VCO1 and O139 Bengal. [13] found that Mathbaria aquatic environment of Bangladesh to be reservoir for *V. cholerae* O1 and O139 Bengal. He also observed that significant clumping of the bacteria during the inter-epidemic period for cholera and the fluorescent micrographs revealed large numbers of *V. cholerae* O1 in thin films of exopolysaccharides (biofilms). They also observed a similar clumping of *V. cholerae* O1 in samples collected from Matlab, Bangladesh, where cholera is also endemic.

CONCLUSION

The current study revealed that there is a correlation between pathogenic *Vibrio cholerae* and zooplankton 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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