# Seasonal dynamics of Vibrio cholerae and its phages in riverine ecosystem of Gangetic West Bengal: cholera paradigm

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# Seasonal dynamics of *Vibrio cholerae* and its phages in riverine ecosystem of Gangetic West Bengal: cholera paradigm

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**Abstract** The Gangetic delta is a century-old cholera endemic belt where the role of riverine–estuarine ecosystem in cholera transmission has never been elucidated. Seasonality, distribution, and abundance of environmental *Vibrio cholerae* O1/O139 and vibriophage in Hooghly riverine–estuarine environment and their correlation with cholera incidence pattern in West Bengal, India, have been analyzed for the first time across summer, monsoon, and winter months. A total of 146 water samples collected from two sites of the Hooghly River (Howrah and Diamond Harbour) were analyzed physicochemically along with cultivable *Vibrio* count (CVC), *V. cholerae* O1/O139, and vibriophages. *V. cholerae* O1 was detected in 56 (38.3 %) samples, while 66 (45.2 %) were positive for *V. cholerae* O1 phages. Flood tide, water temperature ($31 \pm 1.6$ °C), and turbidity ($\geq 250$ nephelometric turbidity unit (NTU)) significantly stimulated *V. cholerae* and vibriophage abundance in riverine ecosystem. Solitary existence of *V. cholerae* O1 and phages ($p < 0.0001$) in aquatic environment divulges the dominance of either of the entity (*V. cholerae* O1 or *V. cholerae* O1 Φ) on the other. Significant association ($p < 0.05$) between Kolkata cholera cases and *V. cholerae* O1 in aquatic environment implies the role of riverine–estuarine ecosystem in cholera transmission. A "biomonitoring tool" of physicochemical stimulants, tidal, and climatic variants has been proposed collating *V. cholerae* and phage dynamics that can forewarn any impending cholera outbreak.

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## Introduction

Cholera epidemics occur with seasonal regularity in the Gangetic delta of India (Colwell 1996). Epidemics generally recur twice a year (Alam et al. 2011), with the highest number of cases in the month of July–October (WHO 2010). Cholera epidemics, caused by toxigenic *Vibrio cholerae* O1 or O139, are a major public health problem in many developing countries (Bhowmick et al. 2009). *V. cholerae* O1 is further classified into two major serotypes, Ogawa and Inaba. Although *V. cholerae* is a human pathogen, they constitute part of the normal aquatic flora also in ponds, lakes, riverine–estuarine environments, etc. (Paul et al. 2012), and water is the established transmission vehicle (Palit and Batabyal 2010; Seed et al. 2012). In spite of lacking conclusive evidences, cholera seasonality in the Indian subcontinent has been associated with numerous physical (temperature, salinity, sunlight, pH, etc.) and biological (phytoplankton, zooplankton, etc.) attributes (Lipp et al. 2002). Conditionally, viable environmental cells (CVEC), alternatively called viable but nonculturable cells, also play a crucial role in cholera epidemiology (Kamruzzaman et al. 2010).

Bacteriophages are known to be an abundant and ubiquitous component of the microbial communities of surface water (Bergh et al. 1989). They are believed to play a significant role in bacterial mortality in surface waters (Jiang et al. 1992). Bacterial viruses are known to play a critical role in the evolution of pathogenic bacterial species and *V. cholerae* in particular (Waldor and Mekalanos 1996). Earlier studies (Das et al. 2009) showed a correlation between *V. cholerae* O1 and its phages in different environmental water samples of natural water bodies. Because of its stability and less sensitivity, vibriophages appear to be better indicators and suggested to be used as a biomonitoring agent in different aquatic ecosystems.

Notwithstanding the above facts, there is a lack of systematic studies to evaluate the seasonal dynamics of both vibriophages and *V. cholerae* O1 in the flowing riverine–estuarine ecosystems of southeastern West Bengal, India, where cholera is a century-old entity. Thereby, implications and correlations of environmental factors and vibriophages with the occurrence of the pathogenic bacteria and resultant effect on cholera incidences have never been elucidated.

The present study has therefore been undertaken to explicitly address the seasonality of *V. cholerae* O1 along with vibriophages, their competitive relationship under regulating environmental factors in Gangetic riverine–estuarine ecosystem, and resultant correlation with disease incidence pattern in the nearby human communities.

## Materials and methods

Two sampling sites in the Hooghly River, branch of the Gangetic riverine system in South Bengal (cholera-prone zone), were selected:

Site 1: "Howrah" sampling site is located in an urban area (130 km inland from sea mouth; 22° 35′ 6″ N, 88° 20′ 49″ E) of the Hooghly River flowing alongside the densely populated cities of Kolkata and Howrah (Fig. 1). Apart from drinking purposes, river water is accessed for multiple usages, viz., washing, bathing, cleaning utensils, etc.

Site 2: "Diamond Harbour" is situated in a rural setup, (80 km inland from sea mouth; 22° 11′ 37″ N, 88° 10′ 48″ E). Being nearer to the sea mouth, the Hooghly River at Diamond Harbour is expectedly wider with higher turbulence and reflects greater intrusion of sea water during tidal oscillations (Fig. 1).

The present study was conducted between January and December 2011, comprising of all the three prevalent tropical seasons of summer, monsoon, and winter to determine the yearlong seasonal and ecohydrological influence on disposition of *V. cholerae* O1 and their phage in riverine–estuarine ecosystem.

Synchronous samplings were carried out at both sites every 3 weeks at an interval of every 2 h, collecting on an average five samples per site per day, from the midstream of the river. Water samples were collected with a sterile metallic bucket (5 L) from about 50 cm underneath the water surface, pooled in a sterile jerry cane (10 L), taken into 500-mL sterile glass bottles, and stored with ice packs in dark Styrofoam boxes. Water temperature, pH, turbidity, conductivity, and salinity were determined immediately after collection using appropriate probes (WTW, Weilheim, Germany).
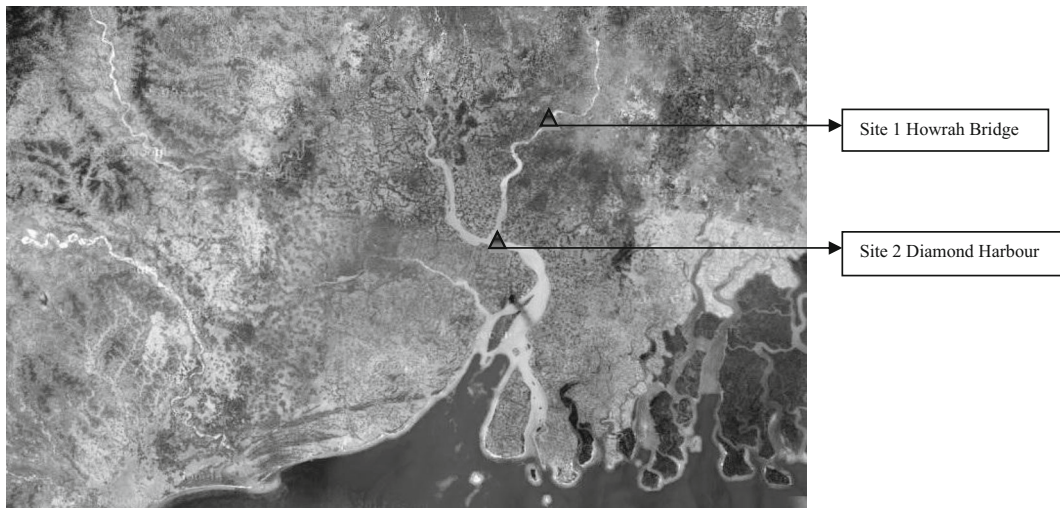
**Fig. 1** Study sites on the Hooghly River

Microbiological analysis

Raw water samples (500 μL) were directly plated on thiosulfate citrate–bile salts–sucrose agar (TCBS, Difco, Sparks, MD, USA) plates, followed by an incubation period of 18–24 h at 37 °C for estimating the cultivable *Vibrio* count (CVC) in colony-forming units (CFU mL$^{-1}$). Simultaneously, sample enrichment and subsequent isolation as well as identification of *V. cholerae* were accomplished following standard protocol (Batabyal et al. 2012). Further, *V. cholerae* isolates were analyzed by serum agglutination test using *V. cholerae* O1 Poly, Ogawa, and Inaba antisera (BD-Difco) and "Bengal O139" antisera (Denka Seiken, Japan).

MPN-PCR technique

Two 50-mL centrifuge tubes were filled with water sample and centrifuged at 11,200×g for 10 min. Following this, 45 mL supernatant from each tube was discarded. The pellets were resuspended, using distilled water, with a vortex mixer. Next, 5 mL aliquot from each centrifuge tubes was mixed with 90 mL Tryptic Soy Broth (TSB, BD) with 1 % NaCl and stomached for 2 min using a stomacher (Remi, India) to homogenize the solution. Salt Polymyxin (Oxoid) was used for dilutions of 100-fold and 1,000-fold of the stomacher fluid prior to most probable number (MPN), and 1 mL of each dilution was pipetted into three tubes and incubated at 37 °C for 18 to 24 h.

Microbial DNA from the MPN tubes was extracted following boiled cell method, as described by Tunung et al. (2009) and Kawasaki et al. (2005). MPN tubes that turned turbid after incubation were centrifuged at 13,400×g for 1 min; the supernatants were discarded and 500 μL of distilled water was added to the tubes to resuspend the pellet. Following this, boiling and immediate cooling of the tubes were done, each for 15 min, respectively. Finally, the tubes were centrifuged again at 13,400×g for 3 min. The clear supernatants were transferred into sterile new microcentrifuge tubes and preserved at −20 °C.

*V. cholerae* O1 and O139 specific primer pairs (Hoshino et al. 1998) were used to detect *V. cholerae* O1 and O139. Amplification of target gene using DNA template was performed using a thermal cycler (Applied Biosystems 2720 Thermal Cycler, USA). Three microliters of boiled DNA from the MPN tubes was added to 25-μL PCR mixture comprising 2.5 μL of 10× PCR buffer, 25 mM of MgCl$_2$, 2.5 μL of 25 mM dNTPs mix, 1.5 μL of each primer, and 1 U of *Taq* polymerase. Amplification conditions were 5 min at 94 °C for predenaturation, followed by 35 cycles of denaturation at 94 °C for 90 s, annealing at 55 °C for 90 s, extension at 72 °C for 90 s, and a final round of extension at 72 °C for 7 min. The PCR products were electrophoresed on 1 % (*w/v*) agarose gel in 1× TAE buffer at 100 V and visualized under ultraviolet light using a computer software (DNr, MiniLumi, Israel). *V. cholerae* O1 isolates were characterized by multiplex PCR for detection of

*tcp* (classical and El Tor) and toxin genes (*ctx*) (Keasler and Hall 1993).

### *V. cholerae* O1 and O139 phage enumeration

*V. cholerae* O1 and O139 phage identification and enumeration were performed as per standard protocol (Das et al. 2009; APHA 2001) with certain modifications, viz., increase in the sample volume up to 100 mL. Briefly, 100 mL of water sample was filtered through a 0.22-μm pore size membrane filter (Millipore, Bangalore, India), and the filtrate was mixed with 20 mL of 5 M NaCl (Sigma, St. Louis, USA). After 1 h at 4 °C, the mixture was resuspended with polyethylene glycol (PEG, Sigma, St. Louis, USA) 8000 (one-tenth volume) and incubated overnight at 4 °C. Then, the phage particles were precipitated by centrifugation at 13,000×*g* for 20 min. Phage pellets were resuspended in 5 mL of Tris–HCl buffer containing $MgCl_2$ and kept at 4 °C. Standard propagating strains were used to determine the presence of environmental *V. cholerae* O1 phages using standard protocol (Das et al. 2009; APHA 2001). Briefly, logarithmic-phase cells (100 μL) of host bacterial strain and Tris-$MgCl_2$ buffer-suspended phage particles were mixed with 3.5 mL soft agar (nutrient broth containing 0.8 % Bactoagar, Difco, Sparks, MD, USA), and the mixtures were overlaid on nutrient agar plates. After overnight incubation, positive samples for vibriophages were identified by the appearance of plaques which were enumerated to estimate the load of *V. cholerae* O1 phage per unit volume of sample.

### Detection of *V. cholerae* conditionally viable environmental cell

The presence of conditionally viable environmental cell (CVEC) of *V. cholerae* O1 was estimated by exploiting the antibiotic resistance property of the bacteria, as described elsewhere (Faruque et al. 2006).

Briefly, an aliquot (5.0 mL) of each water sample was added to 2.5 mL of three times concentrated bile–peptone medium (BP; 1 % peptone, 0.5 % taurocholic acid, 1 % NaCl, pH 9.0) and incubated for 5 h for enrichment of *V. cholerae*. In another set of BP enrichment cultures, an antibiotic, i.e., streptomycin (70 μg mL$^{-1}$) or nalidixic acid (30 μg mL$^{-1}$), was added. Dilutions of the enrichment cultures were spread on taurocholate tellurite gelatin agar (TTGA) plates containing streptomycin (70 μg mL$^{-1}$) and on TTGA plates devoid of any antibiotic. Suspected *Vibrio* colonies were picked and subjected to standard biochemical and serological tests (Kamruzzaman et al. 2010). Samples containing naturally occurring CVEC of recent epidemic strains would not yield *V. cholerae* colonies on TTGA plates if preenriched in the presence of streptomycin but would produce *V. cholerae* colonies when preenriched in the presence of nalidixic acid, although isolated strains exhibit resistance to both these antibiotics.

### Statistical analysis

Results were analyzed applying a "2×2 contingency table" to compare dispositional variation of *V. cholerae* O1 and its phages between two sites (Table 1) and "correlation coefficient" (*r*) to compare the variation of *V. cholerae* O1 and cholera incidence pattern along with physicochemical variables. For estimation of correlation coefficient, "*n*" was always 12. The analyses were done by using Epi Info software (version 3.5.1, USA).

## Results and discussion

Altogether, 146 water samples were collected from the Hooghly River, comprising of 67 samples from Diamond Harbour and 79 samples from Howrah.

### Physicochemical variants

Water temperature varied between 21.1 and 35.4 °C at Diamond Harbour and between 17.3 and 37 °C at Howrah (Table 1), with comparatively higher temperature in summer than in winter season. At Howrah site, the pH of samples ranged between 7.1 and 8.7 and at Diamond Harbour site between 7.1 and 8.1 (Table 1). A more or less consistent trend of higher pH throughout the study period was noted at the Howrah site than that of Diamond Harbour.

At Diamond Harbour, salinity ranged between 0.1 and 7.5 practical salinity unit (PSU) being higher in summer period only. On the contrary, at the Howrah site, salinity always remained steady at values (<0.1 PSU), explaining the long distance of the site (130 km) from the sea mouth. Conductivity ranged between 166 and 619 μS cm$^{-1}$ at Howrah and between 213 and 12,790 μS cm$^{-1}$ at Diamond Harbour (Table 1, Fig. 2). A perceptible increasing trend of conductivity level in summer, at both

**Table 1** Seasonal variation of physicochemical and cultivable *Vibrio* count

| Months | Temperature (°C) | | pH | | Conductivity (μS cm$^{-1}$) | | Turbidity (NTU) | | CVC (CFU mL$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Site 1 | Site 2 | Site 1 | Site 2 | Site 1 | Site 2 | Site 1 | Site 2 | Site 1 | Site 2 |
| Jan | 17.3–18.5 | 21.3–24 | 8.2–8.7 | 7.9–8.1 | 298–350 | 1094–3140 | 150–186 | 126–419 | 0.1–1.5 | 1.8–8 |
| Feb | 23–24.8 | 24.2–26.8 | 7.9–8.1 | 7.5–7.7 | 344–382 | 1500–4200 | 63.5–113 | 30–525 | 2–7 | 1–9 |
| Mar | 26.8–29.9 | 26.1–30.4 | 8–8.3 | 7.7–8.1 | 359–619 | 1788–10200 | 58.1–165 | 33.8–612 | 5.2–14.2 | 0.6–45 |
| Apr | 30.4–32.7 | 30.5–31.5 | 7.7–8 | 7.9–8.1 | 373–386 | 4930–12790 | 166–248 | 266–915 | 4.6–13 | 8.8–176.2 |
| May | 31.3–34.1 | 31.2–34 | 8.1–8.6 | 7.6–7.8 | 359–413 | 4390–7480 | 34.5–106 | 155–391 | 3–26 | 2–7 |
| Jun | 30.1–37 | 29.9–34.8 | 7.4–8.1 | 7.7–8.1 | 166–313 | 470–5950 | 38.1–370 | 194–635 | 16–135 | 8–240 |
| Jul | 30.3–31.6 | 29.3–31.8 | 7.2–7.7 | 7.4–7.8 | 188–220 | 244–440 | 179–245 | 95.2–126 | 47–64 | 1–5 |
| Aug | 30.7–32.6 | 30.1–32.1 | 7.5–7.8 | 7.1–7.7 | 218–247 | 213–1175 | 79.7–557 | 197–713 | 3–786 | 1–182 |
| Sept | 29.7–32.7 | 29.2–32.3 | 7.1–7.6 | 7.2–7.8 | 211–216 | 213–246 | 162–306 | 82.6–100 | 3.4–18.5 | 2.2–7.6 |
| Oct | 30.2–32.1 | 31.2–35.4 | 7.1–7.3 | 7.7 | 235–240 | 306–695 | 129–308 | 173–352 | 5.2–9.1 | 2.1–10.2 |
| Nov | 26.3–28.9 | 26–31.8 | 7.5–8 | 7.3–7.7 | 290–509 | 555–1476 | 39.4–86.2 | 118–365 | 4.2–9.5 | 1–25.8 |
| Dec | 18–19.8 | 21.1–23 | 7.8 | 7.2–7.5 | 348–359 | 365–602 | 31.5–54.4 | 81–126 | 4–5.8 | 0–3.4 |

study sites, indicated the higher intrusion of marine saline water coupled with increased evaporation at summer time. Due to its proximity, Diamond Harbour received higher amount of estuarine brackish water than Howrah which was reflected by comparing their salinity levels. Turbidity was higher at Diamond Harbour (30 to 915 nephelometric turbidity unit (NTU)) in comparison to Howrah (31.5 to 557 NTU) (Table 1, Fig. 3) which might be due to higher tidal influence aided by wind pressure causing resuspension of the sediments from either side of river banks. However, the seasonal variation of turbidity was different in the study sites. While the Howrah site had its peak in monsoon season (July–August) characterized by runoff of waste material under incessant heavy rain, the Diamond Harbour site showed its peak in summer months (March–April). Moreover, new moon and full moon had greater effects on tidal oscillation resulting in an increase in turbidity level at both the study sites.

Microbiological variants

CVC ranged between 0.1 and 786 CFU mL$^{-1}$ at Howrah and between 0.6 and 240 CFU mL$^{-1}$ at Diamond Harbour (Table 1). In most of the samples, a higher CVC of any particular collection schedule was observed in high tide sampling. We have observed a higher peak of CVC during rainy season at Howrah Bridge (because of the inflow of higher volume of floodwater along with fecal organic debris). On the contrary, the CVC peak was observed during summer at Diamond Harbour, which might be related to the inflow of marine saline water carrying higher numbers of *Vibrio* spp. (Lara et al. 2011).
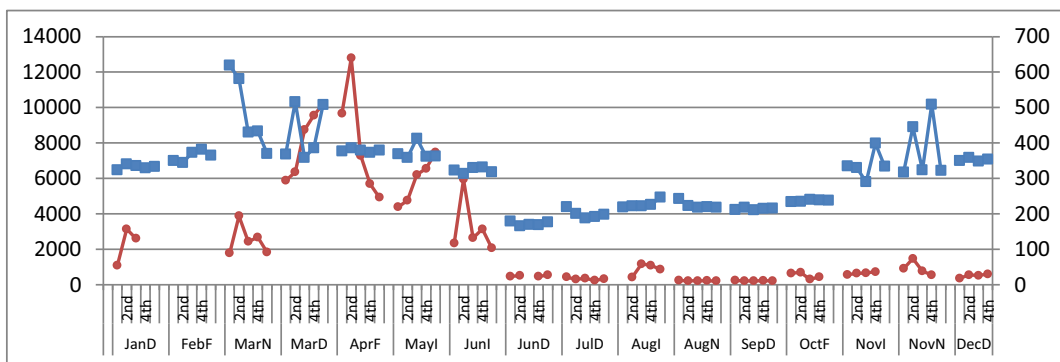


**Fig. 2** Conductivity (μS cm$^{-1}$) level at Howrah (*blue square*; *Y*2 axis) and Diamond Harbour (*red circle*; *Y*1 axis)
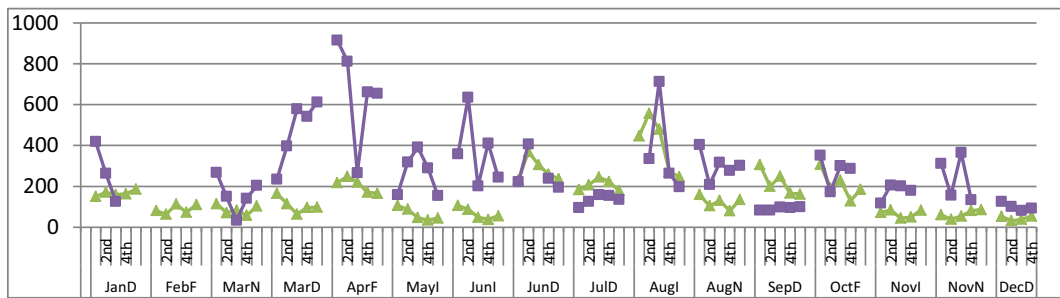
**Fig. 3** Turbidity level (NTU) of water samples at Howrah (*green triangle*) and Diamond Harbour (*violet square*)

Turbidity, among physicochemical variables, showed a better correlation with *Vibrio* abundance ($r=0.8$, $p<0.001$ at Howrah and 0.59, $p<0.05$, at Diamond Harbour) than that of salinity ($r=0.21$, $p<0.2$ at Diamond Harbour) and temperature ($r=0.22$, $p<0.2$ at Howrah and $r=0.4$, $p<0.1$, at Diamond Harbour). Thus, the abundance of free-flowing *Vibrios* in riverine ecosystem at Diamond Harbour during summer is the collective effect of water temperature, turbidity, and salinity (conductivity). Increase in temperature during the onset of summer plays a pivotal role in increasing the free-floating *Vibrios* in the seston fraction (<20 μm) of surface water, and the increase in nutrient sediments may also act synergistically to aid in bacterial growth (Neogi et al. 2011, 2012; Lara et al. 2009). Increase in salinity (conductivity) level at Diamond Harbour especially during March–May (Fig. 2) indicated the higher intrusion of marine saline water bringing in a diversified halophilic bacterial community including the *Vibrios* along with its inflow. Simultaneously, the greater tidal oscillation enabling the resuspension of sediments and attached *Vibrio* community might have resulted in increased turbidity as well as CVC (Table 1, Figs. 2 and 3).

*V. cholerae* O1 and *V. cholerae* O139

More than one third of the samples, i.e., 56/146 (38.3 %), were harboring *V. cholerae* O1, consisting of 49 (87.5 %) Ogawa and 7 (12.5 %) Inaba strains, out of which 34 samples were from Howrah and 22 were from Diamond Harbour. *V. cholerae* O1 ranged between 1 and 15 CFU mL$^{-1}$ at both the study sites (Fig. 4). Despite of our cautious screening attempts, no *V. cholerae* O139 could be isolated from any site during the study period which considerably explains the near total absence of *V. cholerae* O139-induced cholera cases in the surrounding localities.

Although at Diamond Harbour, the trend of increasing preponderance of *V. cholerae* O1 was observed parallel to increase in salinity and temperature during summer period, with an observable peak in April, the peaks of preponderance of *V. cholerae* O1 at Howrah was observed both at the onset of summer (smaller peak) and during monsoon (larger peak) (Table 1, Fig. 4). Abundance of *V. cholerae* O1 from the month of June (summer) to September (monsoon) was probably due to the interactions of intruded water from the downstream region during high tides carrying higher number of halophilic *Vibrios* including *V. cholerae* and runoff of organic debris including fecal waste along with inflowing flooded water (from the adjoining surroundings) due to heavy rainfall (Rainfall data, http://climate.usurf.usu.edu/mapGUI/mapGUI.php).

Altogether, eight *V. cholerae* O1 isolates were detected to harbour *ctx* (major virulence gene) and *tcp* gene of which six were isolated from Howrah and two from Diamond Harbour. Among the potentially toxigenic *V. cholerae* O1, while Diamond Harbour isolates showed their prevalence during summer (April), Howrah isolates were detected during late summer and early monsoon (June–August).

In this yearlong study, *V. cholerae* O1 showed higher correlation ($r=0.7$, $p<0.01$) with turbidity and salinity at the Diamond Harbour site. At the Howrah site, however, *V. cholerae* O1 showed significant correlation ($r=0.5$, $p\leq0.05$) with turbidity only.

*V. cholerae* O1 and *V. cholerae* O139 Φ

A total of 66 (45.2 %) samples were observed to harbour *V. cholerae* O1 Φ, out of which 42 samples were from Howrah and 24 from Diamond Harbour. Higher preponderance of *V. cholerae* O1 Φ was noted during summer (at Diamond Harbour and Howrah) followed by
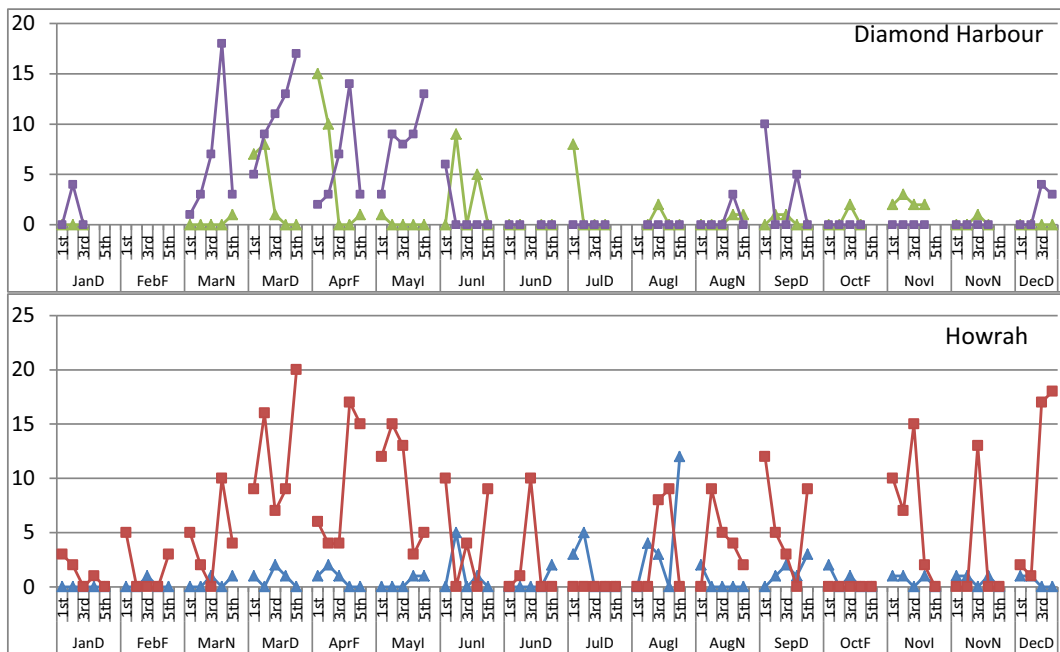
**Fig. 4** Seasonal dispositional variation of *V. cholerae* O1 (CFU mL$^{-1}$; *blue triangle*) and *V. cholerae* O1 Φ (PFU mL$^{-1}$; *red square*)

monsoon (at Diamond Harbour and Howrah) and winter period (only at Howrah). Environmental factors like higher water temperature (during summer) and heavy rainfall-mediated (during monsoon) input of organic sediments including fecal waste were probably the inducing factors for the abundance of *V. cholerae* O1 which seems to regulate the *V. cholerae* O1 Φ load (Fig. 4) also. At both the study sites, *V. cholerae* O1 Φ ranged between 1 and 20 PFU mL$^{-1}$. Like *V. cholerae* O139, no *V. cholerae* O139 Φ could be isolated, which can be related to the near total absence of *V. cholerae* O139 as the causative agent of cholera cases in nearby human localities. Among the etiological agents of cholera, the initial seventh pandemic *V. cholerae* O1 ElTor typical strain and *V. cholerae* O139 have nearly disappeared whereas a *V. cholerae* O1 ElTor variant which is comparatively more virulent has become completely dominant in the Indian subcontinent (Yamasaki et al. 2010).

The coexistence of *V. cholerae* O1 and its phage was observed in the cases of 19.8 % (29/146) samples, whereas almost 42.4 % (62/146) samples were harboring either *V. cholerae* O1 or its phage. Statistically significant distributional variation (*p*<0.0001) between coexistence and solitary existence of *V. cholerae* O1 and its phage indicates the dominance of any one of the entity (either *V. cholerae* O1 or *V. cholerae* O1 Φ) on

the prevalence of the other than that of coexistence as a natural phenomenon. Therefore, we can interpret that phage can be a potential delimiting factor of its host in both freshwater and estuarine environments. It can further be evidentially (Fig. 4) supported by our evaluation of seasonal preponderance of *V. cholerae* O1 and its phages, where most of the *V. cholerae* O1 peak coincided with the minimum load of *V. cholerae* O1 phage and vice versa.

Detection of *V. cholerae* CVEC

Yearlong surveillance of riverine–estuarine environment has revealed that prevalence of CVEC was notably higher in Howrah during summer followed by winter. On the contrary, persistence of CVEC could be observed at Diamond Harbour during winter. Thus, from this seasonality in the prevalence of CVEC, we interpret and infer that the winter season in this tropical environment is conducive for the transformation of viable *V. cholerae* to a conditionally viable one. However, the predominance of CVEC in Howrah during summer indicates the intrusion of *V. cholerae* O1 pool from Diamond Harbour to the inland aquatic condition (Howrah) through tidal oscillation enabling them to transform into CVEC condition with salinity variation ranging from 7.5 ppt (at Diamond Harbour) to as low as

<0.1 ppt (at Howrah). Thus, the observation of CVEC in study sites explains the collating induction of environmental factors like salinity, water temperature, etc. on environmental pool of *V. cholerae* O1.

Disease surveillance data in Kolkata have very interestingly shown a marked seasonality in the numbers of cholera cases attributed to *V. cholerae* O1 (You et al. 2013). Significant association ($p < 0.05$) has been observed between Kolkata cholera case seasonality and *V. cholerae* O1 load in aquatic environment in our observation. The prevalence of *V. cholerae* O1 at the two study sites appears to have a direct consequential impact on Kolkata cholera incidences (Fig. 5).

While the first cholera peak could be observed during summer (April), the second peak has been observed in monsoon. During the late summer and monsoon, the preponderance and isolation of *V. cholerae* O1 can also very nicely be extrapolated on seasonal cholera outbreaks (You et al. 2013) in this part of the Indian subcontinent especially in and around the endemic belt of the Gangetic delta of West Bengal (Fig. 5). However, the observed cholera peak in early summer, without any parallel remarkable peak in *V. cholerae* O1 load in riverine system at Howrah site, indicates the silent role of quiescent form of aquatic CVEC of *V. cholerae* O1 community, which when gains passage through human host attains critical phenotypic properties that enhance the infectivity of *V. cholerae* O1 as demonstrated earlier by Merrell et al. (2002). Adding to this, the role of CVEC *V. cholerae* O1 can also be observed during winter, when a small but observable peak of cholera cases has been reported despite of the absence of cultivable *V. cholerae* O1 from our environmental surveillances. The observed peak of CVEC during winter and summer can further be supported by the fact of depletion of nutrient in aquatic environment (Alam et al. 2007).

It can thereby also be evidentially corroborated with our present environmental *V. cholerae* dynamics, e.g., absence of *V. cholerae* O1-specific phage in all the observed samples during monsoon (specially between June to August) in both the study sites (Fig. 4) and simultaneous increase in the pathogenic *V. cholerae* O1 population and resultant interpretation of *V. cholerae* O1—phage dynamics. Therefore, the active role of *V. cholerae* O1 phage as a delimiting factor (Faruque et al. 2005) that neutralizes the *V. cholerae* O1 load to a certain low level (bacteria–phage interaction) has been positively correlated, and its subsequent implications have been evidentially demonstrated by us for the first time. However, occasional increase in abundance of *V. cholerae* O1 signifies the possible incapacitation of *V. cholerae* O1 Φ to control the population of *V. cholerae* O1 during monsoon months (between June and August) which possibly results in seasonal cholera outbreaks. This observed aquatic environmental dynamics of *V. cholerae* O1 and its phages can therefore collate towards a package of forewarning "predictive biomonitoring indicators" for a future impending cholera outbreak that can be caused by direct exposure of the neighboring population to river water through its access and daily usage.

Environmental surveillance by simultaneous monitoring of *V. cholerae* and its phage has so far been a nonentity in the Gangetic delta of eastern India (West Bengal), where epidemic outbreaks and endemicity of cholera have exhibited a seasonal pattern characterized by fluctuation of cholera incidences (Fig. 5).

All previous six cholera pandemics started from the Ganges delta of Bengal. During the seventh pandemic (which started from Indonesia), cholera was prevalent in Bengal and is still endemic in the entire focus (Islam et al. 1993). Cholera has always continued to be recognized in the Indian subcontinent (Harris et al. 2012) including
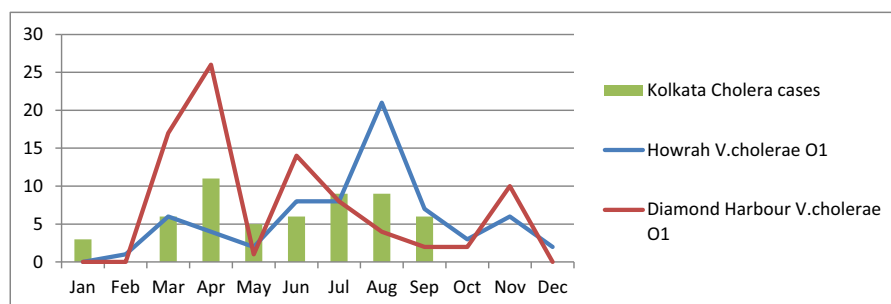


**Fig. 5** Seasonal dispositional variation *V. cholerae* O1 in riverine–estuarine environment and seasonal variation of cholera cases in and around Kolkata City in 2011 (You et al. 2013)

West Bengal, India. It has been endemic through centuries from the very beginning of its history being explored, irrespective of social and political changes (preindependence British colonial regime or postindependence era) and large-scale economical progress albeit in an uneven way leading to sustenance of the disease with conditions remaining untouched by historical phenomena.

However, it is worthwhile to mention here that the kind of social stigma collated with political and economic instability as encountered in Peru (Jones et al. 2008) or Argentina (Carbonetti et al. 2007) during epidemic management in a rapid response situation has never been the same in Indian context, where government machinery, notwithstanding political lineages, steps up preventive machinery. The identified indices can therefore be combined for a suitable "model" for many developing countries worldwide, where similar public health management system exists.

Our endeavor was to respond to long-standing queries regarding the environmental mechanism by which the endemicity of cholera of the Ganges delta is maintained and how this delta remains a reservoir for cholera infection. To our existing knowledge, this is the first longitudinal predesigned systematic study report in the dynamic Hooghly riverine–estuarine aquatic environment, addressing the seasonality of *V. cholerae* O1/O139 along with its phages and parallely correlating their abundances with environmental factors in relation to cholera dynamics. As evident from the present study, in a dynamic aquatic riverine setting like the Hooghly River, a tributary of the Gangetic riverine system, *V. cholerae* O1 and its phages in association with specific physicochemical indicators, viz., water temperature, salinity (or conductivity), and turbidity, are a potential cluster of indicators collating an effective biomonitoring tool for identifying, correlating, and understanding environmental *V. cholerae*-induced cholera dynamics that will be of immense implications in future cholera outbreak prediction.

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