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Seasonality and toxigenicity of *Vibrio cholerae* non-01 isolated from different components of pond ecosystems of Dhaka City, Bangladesh

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Diarrhoea due to *Vibrio cholerae* non-01 is common in Bangladesh. Four hundred and eighty samples, including plants, water, phytoplankton and sediment, were collected from five ponds in Dhaka every 15 days for one year. *V. cholerae* non-01 was isolated from 181 (38%) of the samples. Two peaks were evident: one in April and the other in August/September. Forty-three (23%) of the 181 isolates were examined for toxigenicity and 19% were cytotoxic to Y1 adrenal cells. This study provides evidence of the likely infectious nature of some ponds and may have relevance to the epidemiology of diarrhoea caused by *V. cholerae* non-01 in Bangladesh.

Key words: Diarrhoea, seasonality, toxigenicity, Vibrio.

Vibrio cholerae non-01 is a causative agent of moderate-to-severe, cholera-like diarrhoea in Bangladesh and other parts of the world (McIntyre *et al.* 1965; Zafari *et al.* 1973; Hughes *et al.* 1978). Strains of *V. cholerae* non-01 are morphologically and biochemically similar to those of *V. cholerae* 01 but are distinguished by lack of agglutination with group 01 antiserum (Colwell *et al.* 1977). Food-borne and water-borne outbreaks due to *V. cholerae* non-01 have been reported from various countries, including Czechoslovakia, Sudan, Saudi Arabia and India (Aldova *et al.* 1968; Zafari *et al.* 1973). Epidemics due to *V. cholerae* non-01 have also been reported during cholera outbreaks (McIntyre *et al.* 1965; Dutt *et al.* 1971). These organisms have been isolated from septicaemic and meningoencephalitis patients (Prats *et al.* 1975).

The enterotoxigenicity of *V. cholerae* non-01 demonstrated in the rabbit ileal loop (RIL) assay and by other methods indicates that these strains are potential pathogens (Zinnaka & Carpenter 1972). The enterotoxin produced by *V. cholerae* non-01 is closely related to cholera toxin produced by classical *V. cholerae* 01 (Kaper *et al.* 1979).

The ecology of *V. cholerae* non-01 has been studied in the Chesapeake Bay and California coastal waters (Colwell *et al.* 1977; Kenyon *et al.* 1984). These studies demonstrated a link between the number of organisms and water temperature. In Bangladesh, Khan *et al.* (1984), in investigating the role of surface water as a possible reservoir of *V. cholerae* non-01 in the environment, found the bacteria in approximately 30% of samples tested. However, other components such as plants, plankton and sediment were not evaluated as possible reservoirs nor was the seasonality of the organisms documented. Moreover the organisms recovered were not examined for virulence factors. Therefore, in the present study we examined the seasonality of *V. cholerae* non-01 in the various components of the aquatic environment in addition to surface water and evaluated the toxigenicity of the isolated strains.

Materials and Methods

Description of Ponds

The surface area and depth of all ponds ranged from 1900 to 6000 m^2 and 1.5 to 5.2 m, respectively. The water temperatures varied from 18 to 34°C and the salinities from 0 to 0.18‰. In all except pond No. 1, the waters were alkaline and used by large numbers of people every day for bathing and washing. All ponds were over-flooded during the 1988 flood.

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Collection of Samples

Samples of water, plants, phytoplankton and sediments from five ponds in and around Dhaka city, Bangladesh, were collected every 15 days, between May 1988 and April 1989. Plants (*Eichhornia crassipes, Nymphoides* sp. and *Telanthera philoxeroides*) were collected and kept in polyethylene bags. Samples of water, each of 500 ml, were collected in pre-sterilized 500 ml narrow-mouth, plastic bottles. Phytoplankton was collected using plankton nets and kept in 120 ml glass bottles. All field samples were transported to the laboratory inside an insulated ice-box with cool packs and processed within 6 h of collection. Sediment was collected with a core sampler and kept in 120 ml glass bottles.

Processing of Samples

Plant material (10 g) was homogenized in 100 ml of sterile 0.9% saline in a Waring blender. Phytoplankton samples (10 ml) were homogenized in a Teflon-tipped tissue grinder (Wheaton Scientific, Millville, NJ) using a Sted Fast stirrer (Model 300, Fisher Scientific, USA). One ml of the plant homogenate, 10 ml of phytoplankton homogenate, 50 ml of each water sample and 1.0 g of sediment were enriched separately in bile salts peptone broth and incubated overnight at 37°C. All samples were then plated onto thiosulphate citrate bile salts sucrose (TCBS) agar and taurocholate tellurite gelatin agar and incubated at 37°C for 18 to 24 h (Monsur 1961). Suspected vibrio colonies were further characterized using standard procedures (West & Colwell 1984; Lee 1985).

Enterotoxin Assays

Bacterial Isolates. Forty-three isolates of *V. cholerae* non-01 were selected randomly and tested for toxigenicity using three assays. The strains were isolated from plants, water, phytoplankton and sediment samples.

Preparation of Culture Filtrates. The test strains were each grown in 10 ml of Richardson's medium in 50 ml conical flasks (Richardson 1969). The flasks were incubated in a shaking water bath with 100 rev/min for 18 h at 37°C. The cultures were centrifuged at 10,000 × ***g*** for 30 min. The supernatants were filtered through 0.22 μm pore membrane filters and stored at −20°C for use in the following assays.

Rabbit Ileal Loop (RIL) Assay. One ml of culture filtrate from each of the test strains was inoculated into a 4 to 6 cm length of ileal loop from 1.5 to 2.0 kg New Zealand white rabbits (De & Chatterjee 1953). Each strain was tested in duplicate. *Vibrio cholerae* 01 strain 569B was used as a positive control. Eighteen hours after inoculation, animals were sacrificed and the results were expressed as the accumulated fluid volume in ml per cm of loop.

G_{M1} ELISA. The presence of cholera toxin was assayed using the G_{M1} ganglioside ELISA according to the method described by Sack *et al.* (1980).

Y1 Adrenal Cell Assay. The filtrates were added to Y1 adrenal cells plated in 96-well plates 24 h previously and incubated at 37°C in 7% CO_2, as described by Sack & Sack (1975). After 24 h, cells were examined for cytotoxic and cytotonic effects.

Results

Vibrio cholerae non-01 was isolated from 181 (38%) of the 480 environmental samples collected (Table 1), of which pond No. 5 had the highest isolation rate of 62 from 96 samples collected (65%). In contrast, no organisms were recovered from any of the 96 samples collected from pond No. 1.

Pond No. 5 also yielded most organisms in each of the four sample types: 87% of water samples, 80% of phytoplankton, 67% of the plants and 25% of the soil sediment samples were positive (Table 1).

The toxigenicities of 43 strains randomly selected for testing are shown in Table 2. Twelve strains isolated from plants, 12 from water, 11 from phytoplankton and eight from soil were tested. Overall, eight strains (19%) were cytotoxic for Y1 adrenal cells. No cytotonic activity was detected in the Y1 adrenal cell assay. All strains were also negative in the G_{M1} ELISA and RIL assays.

Distinct seasonal patterns of isolation of *V. cholerae* non-01 in the four components of the aquatic ecosystems were observed. Two peaks were detected; the highest peak occurred in April in all four components and the second, smaller peak, occurred in August in plants and phytoplankton and in September in water and sediment (Figure 1). A marked decline in isolation rate occurred in components other than water, in the month of July.

Table 1. Abundance of *V. cholerae* non-01 in different components of the ponds.

Pond no.	Sample									
	Plant		Water		Phytoplankton		Soil sediment		Total	
	NP/NT	(%)	NP/NT	(%)	NP/NT	(%)	NP/NT	(%)	NP/NT	(%)
1	0/24	0	0/24	0	0/24	0	0/24	0	0/96	0
2	7/24	29	21/24	88	17/24	71	4/24	17	49/96	51
3	7/24	29	18/24	75	12/24	50	5/24	21	42/96	44
4	4/24	17	13/24	54	8/24	33	3/24	13	28/96	29
5	16/24	67	21/24	88	19/24	79	6/24	25	62/96	65
Total	34/120	28	73/120	61	56/120	47	18/120	15	181/480	38

NP—Number of samples positive for *V. cholerae* non-01. NT—Number of sample tested.

Table 2. Enterotoxigenicity tests with culture filtrates of *V. cholerae* non-01.

Source of isolates	Y1 adrenal cell assay		RIL assay & G_{M1} ELISA	
	No. of filtrates cytotoxic/ No. tested	Positive (%)	No. positive/ No. tested	Positive (%)
Plants	2/12	17	0/12	0
Water	2/12	17	0/12	0
Phytoplankton	2/11	18	0/11	0
Soil	2/8	25	0/8	0
Total	8/43	19	0/43	0

Discussion

The present investigation shows the seasonality of *V. cholerae* non-01 in plants, water, phytoplankton and soil sediment in four of five pond ecosystems in and around Dhaka city. The highest peak in isolation rates in all four components was during the summer. Our results agree with other studies conducted in England and U.S.A. (Blake *et al.* 1980; Lee *et al.* 1982). The presence of the *V. cholerae* non-01 in various components of pond ecosystems indicates that freshwater ponds may act as a reservoir for *V. cholerae* non-01 in the aquatic environment. The role of plants and phytoplankton in the survival and transmission of *V. cholerae* has been studied previously (Islam & Aziz 1981; Islam *et al.* 1984, 1989, 1990a,b). The presence of the bacteria in the ponds throughout most of the year may contribute to the endemicity of *V. cholerae* non-01 in Bangladesh. Khan *et al.* (1984) noted a correlation between the rate of isolation of *V. cholerae* non-01 from the aquatic environment in Dhaka and the incidence of *V. cholerae* non-01 diarrhoea treated in the local Clinical Research Centre.

In contrast to ponds 2 to 5, *V. cholerae* non-01 was not isolated from pond No. 1 during the study period, which may be due to its acidic pH and low salinity.

It is unclear if all *V. cholerae* non-01 strains are enterotoxigenic (Huq *et al.* 1980; Madden *et al.* 1981). Other virulence factors such as cytotoxicity and presence of permeability factors and haemolysins can also occur in these bacteria. Only a proportion of *V. cholerae* non-01 produce cholera-like toxin (Kodama *et al.* 1984); according to the World Health Organization (1984), this toxin is produced by less than 10% of *V. cholerae* non-01 isolated from clinical sources and less than 1% of isolates from the environment. None of the 43 strains selected in the present study for testing demonstrated cholera toxin-like activity, which is in agreement with other studies (Madden *et al.* 1981). Kaper *et al.* (1979) demonstrated that 87% of the *V. cholerae* non-01 strains isolated from the Chesapeake Bay exhibited cytotoxic activity in Y1 adrenal cells, compared with 18.6% of strains in our study.

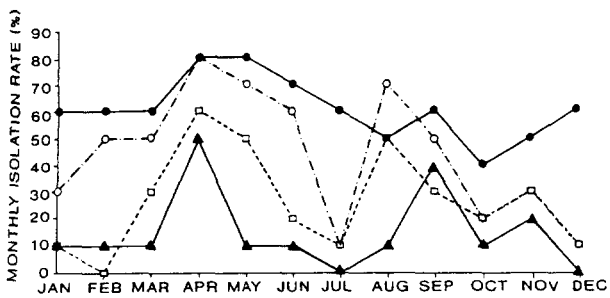

Figure 1. Seasonal changes in isolation rate (%) of *V. cholerae* non-01 from water (●), phytoplankton (○), soil sediment (▲) and plant (□) samples.

The presence of *V. cholerae* non-01 in all components of pond ecosystems most of the year demonstrated that untreated water from these sources was unfit for human consumption. Yet these water sources are routinely used for cooking, bathing and washing by local people. Studies in Bangladesh have shown that the use of safe drinking water from tube-wells did not reduce the incidence of cholera infection and this was attributed to the use of contaminated surface water (Sommer & Woodward 1972).

The persistence of *V. cholerae* non-01 in ponds which are extensively used indicates a possible source of infection. The increased incidence of diarrhoea in the summer may be related to the observed increase in vibrio populations in the ponds at the time. The persistence of vibrios in the environment and the yearly recurrence of outbreaks of disease have never been adequately explained. Our study, by defining some of the ecological niches exploited by *V. cholerae* non-01, may begin to identify important links in the pathways of transmission of these organisms. This study gives some insight into the risk of contracting infections from aquatic environments and has relevance to the epidemiology of *V. cholerae* non-01 diarrhoea in Bangladesh.

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