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Incidence and toxigenicity of *Vibrio cholerae* in a freshwater lake during the epidemic of cholera caused by serogroup O139 Bengal in Calcutta, India

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Abstract: The extent of contamination of a freshwater lake with *Vibrio cholerae* O139 Bengal and the toxigenicity of all the *V. cholerae* isolates recovered during the period of the study were examined during and after an explosive outbreak of O139 cholera in Calcutta. Strains biochemically characterized as *V. cholerae* could be isolated throughout the period of study examined from the freshwater lake samples. Most probable number of *V. cholerae* belonging to the O139 serogroup in surface waters was 3 to 4 per 100 ml during major part of the study but isolation of this serogroup from sediment and plankton samples was infrequent. Of the total of 150 strains recovered, 23 (15.3%) agglutinated with the O139 antiserum while the remaining belonged to the non-O1 non-O139 serogroups. None of the strains agglutinated with the O1 antiserum. All the 23 strains of *V. cholerae* O139 produced cholera toxin while 7.9% of the 127 non-O1 non-O139 strains also produced cholera toxin. Resistance to ampicillin, furazolidone and streptomycin was encountered among strains belonging to both *V. cholerae* O139 and *V. cholerae* non-O1 non-O139 strains, but the percentage of resistant strains in the former was much higher than in the latter. During this cholera epidemic, possibly due to the introduction of large numbers of toxigenic *V. cholerae* such as the O139 serogroup, there was an increase in the numbers of toxigenic vibrios among the innocuous aquatic residents. This presumably occurred through genetic exchange and, if substantiated, could play an important role in the re-emergence of epidemics.

Key words: Vibrio cholerae O139 Bengal; Cholera toxin; Environmental distribution during cholera epidemic

Introduction

Cholera continues to be an important and common cause of potentially fatal dehydrating diarrhoea, particularly in adults, in Calcutta and other cholera-endemic areas in India. Despite significant advances in the understanding of the disease, the role of natural aquatic realms in the transmission of cholera and in sustaining the O1 serogroup of *Vibrio cholerae* (until recently the only causative serogroup of epidemic and pan-

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demic cholera) remains uncertain. *V. cholerae* that do not belong to the O1 serogroup, collectively referred to as non-O1 *V. cholerae*, are ubiquitous residents of aquatic environs [1,2]. In contrast, the isolation of 'culturable' *V. cholerae* belonging to the O1 serogroup from the environment are few and far between, and those that have been isolated are invariably non-toxigenic [3]. The incidence of 'culturable' toxigenic *V. cholerae* O1 in aquatic environs coincides with the incidence of active cholera cases in the vicinity or during periods of epidemics [1,4,5]. Increasing evidence seems to suggest that, during the inter-epidemic period, the O1 serogroup of *V. cholerae* reverts into a viable but non-culturable state [6].

A novel strain of *V. cholerae* that does not agglutinate with the O1 antiserum, classified as serogroup O139 Bengal [7,8], has been responsible for extensive outbreaks of cholera in several countries in south Asia since October 1992 [9–12] and is believed to have originated from Madras in southern India [13]. Like the O1 serogroup of *V. cholerae*, the O139 serogroup produces cholera toxin [14]. The epidemic spread of cholera has been linked with the widespread contamination of aquatic environments as has been reaffirmed during the recent extension of the seventh pandemic of cholera in Latin America [15]. In Calcutta, a large outbreak of cholera caused by the O139 serogroup occurred from mid-February 1993 and continued until the end of June 1993 [13,16]. During and after the period of the epidemic caused by the O139 serogroup, we conducted an investigation to study the extent of contamination of the aquatic environs with *V. cholerae* and to determine the toxigenic status of these environmental isolates in Calcutta.

Materials and Methods

Sampling area

Subhash Sarobar, a man-made lake occupying an area of 39.5 acres, is located in the eastern part of Calcutta (longitude 88°20′E; latitude 22°32′N), a city located in the north-eastern part of India. Residents living in the vicinity of the lake utilize the lake water for bathing, washing clothes and utensils and for ablution purposes but the lake does not receive any industrial or domestic sewage. Five sites located either near or at the bathing sites in the lake were selected for sampling. Quantitative analysis was performed only at site 2, while at sites 1, 3, 4 and 5 only qualitative analysis of water, sediment and plankton samples was performed. Starting from April 2 to August 19 1993, sampling was done twice a week for a month, weekly for the next month and then fortnightly for the remaining period.

Collection of environmental samples

Water, sediment and plankton samples were collected as described elsewhere [17]. Planktonic organisms were harvested by towing a standard horizontal plankton net (bolting silk no. 20; mesh size 77 μm) for ca. 30 min at sub-surface level. Pre-sterilized glass flasks were used to collect water while sediment samples were collected using a Petersen grab. At the laboratory, sediment samples were aseptically weighed into sterile collection area water and mechanically stirred for 15 min to dissociate the aggregated bacterial population. The settlings of the agitated sample was analyzed bacteriologically.

Physical parameters

Methods used for the measurement of temperature and pH were as described previously [18].

Bacteriology

The method used for enumeration of *V. cholerae* was the three tube most-probable-number (MPN) procedure described by Kaper [19]. Appropriate decimal dilution ranging between 1 and 10^{-6} using sterilized water samples from the collection area as diluent were used. Alkaline peptone water (APW, pH 8.5) was used as the enrichment broth. Two or three loopfuls of cultured broth (APW) were plated on thiosulfate-citrate-bile-salt sucrose agar (TCBS; Eiken Chemical Co. Ltd., Tokyo, Japan) for selective isolation of *V. cholerae*. After overnight incubation at 37°C, typical sucrose-fermenting colonies were inoculated onto a multi-test medium to facilitate rapid presumptive identification of *V. cholerae*. Strains that showed alkaline slant and

acid butt reaction without gas production in the multi-test medium [20] were examined for the oxidase reaction. MPN values of samples yielding confirmed isolates were determined using the appropriate formula as described [18].

Serology

Slide agglutination was performed with polyvalent O1 and monospecific Ogawa-Inaba antisera prepared at the Institute. Strains which did not agglutinate with the O1 antiserum were examined for agglutination with O139 antiserum prepared by hyperimmunizing rabbits with heat-killed whole cells of reference strain MO45 (ATCC 51394) of *V. cholerae* O139 [8]. The O139 antiserum was then absorbed with the reference R culture (CA385) of *V. cholerae* and the reference strain (169–68) representing serogroup O22 of *V. cholerae* to remove R agglutinins and cross-reacting agglutinins of serogroup O22, respectively [9,14]. Representative strains of *V. cholerae* that did not agglutinate with either O1 or with O139 antisera were grouped by the somatic O antigen serogrouping scheme of *V. cholerae* [9] developed at the National Institute of Health, Tokyo, Japan.

Detection of cholera toxin

Confirmed strains of *V. cholerae* O139 and all strains of *V. cholerae* that did not agglutinate with O1 or O139 antisera were examined for production of cholera toxin (CT) by a highly sensitive bead enzyme-linked immunosorbent assay [21]. The strains were grown in casamino acid yeast extract medium supplemented with 90 μg ml^{-1} of lincomycin (Sigma Chemical Co., St. Louis, MO) using stationary culture at 30°C in 90×16 mm Petri dishes [22]. After incubation, the cultures were centrifuged at 5000 rpm at 4°C for 10 min and the supernatants were used for the detection of CT.

Antibiotic sensitivity testing

Antimicrobial susceptibility of all confirmed *V. cholerae* non-O1 strains was performed on Mueller Hinton Agar (Difco) employing the dry disc diffusion technique [23] and the antimicrobials listed in Table 2.

Statistical analyses

Relationship between the densities of *V. cholerae* in water, plankton and sediment at site 2

Table 1

Variations in pH, temperature and MPN[a] counts of *V. cholerae* at site 2 of Subhash Sarobar

Date of sampling	pH		Temperature (°C)			MPN [a]								
	Water	Sediment	Water	Sediment	Atmospheric	Water/100 ml			Sediment/100 g			Plankton/g		
						VCLO [b]	Vc [c]	Vc O139	VCLO	Vc	Vc O139	VCLO	Vc	Vc O139
15.04.93	8.4	6.5	31.0	31.0	33.5	7599	950	0	438	9	3	26	3	0
19.04.93	8.4	6.9	32.0	31.5	33.5	438	15	3	44	23	0	72	9	6
21.04.93	9.3	7.0	32.5	32.0	32.5	116	16	0	19	11	0	15	4	0
26.04.93	9.0	6.8	31.5	31.0	32.0	6	0	0	15	11	0	0	0	0
03.05.93	8.7	7.3	33.0	31.0	33.0	4	4	4	0	0	0	0	0	0
11.05.93	8.0	7.0	26.0	30.0	28.0	95	15	4	76	10	0	15	4	0
17.05.93	9.0	6.9	33.0	31.0	34.0	76	10	4	15	0	0	10	4	0
26.05.93	7.7	6.5	31.0	32.0	33.5	438	15	4	26	11	0	1	0	0
07.06.93	7.5	6.9	31.5	32.0	33.0	26	6	3	30	3	0	1	< 3	0
21.06.93	8.0	6.7	31.0	30.5	32.0	26	11	0	0	0	0	0	0	0
06.07.93	7.6	7.0	32.0	32.5	30.0	20	4	0	0	0	0	0	0	0
21.07.93	8.2	6.5	29.0	32.0	32.0	11	4	0	0	0	0	0	0	0
19.08.93	8.8	6.8	30.5	31.0	29.0	26	11	4	46	0	0	0	0	0

[a] MPN, most probable number.
[b] VCLO, *V. cholerae*-like organism.
[c] Vc, *V. cholerae*.

and the environmental parameters were evaluated by generating a correlation coefficient matrix on a micro computer using an SPSS package version 4.2.

Results

Quantitative studies on the density of *V. cholerae* was performed at site 2 of the freshwater lake. Table 1 presents the variations in physical parameters and the MPN counts of *V. cholerae* at site 2 during the period of study. The pH of surface waters ranged between 7.5 and 9.3 with alkaline values being observed during a major part of the study period while that of sediments ranged between 6.5 and 7.3. Fluctuations in water, sediment and atmospheric temperatures were unremarkable. The MPN counts of strains biochemically characterized as *V. cholerae* showed a declining trend in water samples during the period of study while only slight variations in counts were observed in sediment and plankton samples. MPN counts of *V. cholerae* O139 were < 1 to 4 per 100 ml of water through most of April and May 1993 but could not be isolated during late June and July 1993. The O139 serogroup could again be isolated from water samples at this site on August 19 1993 (Table 1). In contrast to surface water samples, isolation of the O139 serogroup from sediment and plankton samples only occurred in April. No statistical significance between environmental variables and the densities of *V. cholerae* in water, sediment or plankton was discernible.

During the period of study, a total of 102 samples comprising of water, plankton and sediment were examined from the 5 sites in the freshwater lake. Strains confirmed as *V. cholerae* could be isolated from 78.9%, 46.1% and 30.8% of the water, sediment and plankton samples, respectively. *V. cholerae* O139 was most frequently isolated from surface waters (22.4%) as compared to plankton and sediment samples (4.7% each). *V. cholerae* O1 was not isolated during the entire period of the study. A total of 150 strains of *V. cholerae* were recovered in this study, of which 23 (15.3%) agglutinated with the O139 antiserum. None of the *V. cholerae* strains agglutinated with the O1 antiserum. Serogrouping of a representative 23 strains of the remaining 127 strains of *V. cholerae* which did not agglutinate with either the O139 or O1 antisera revealed the occurrence of the following serogroups: six strains belonged to O40, two each to O48 and O95, one each to O2, O8, O14, O19, O32, O41, O92, O103, O124 and 4 strains could not be typed.

All 23 strains of *V. cholerae* O139 isolated from the various samples produced CT as determined by the sensitive bead-ELISA. In addition, 10 (7.9%) of the 127 strains which did not aggluti-

Table 2

Frequency of resistance of *V. cholerae* strains isolated from freshwater lake to various antibiotics

Antibiotic (μg/disc)	Number (%) resistant					
	V. cholerae O139 ($n = 18$)			*V. cholerae* non-O1 non-O139 ($n = 23$)		
	Water ($n = 14$)	Sediment ($n = 2$)	Plankton ($n = 2$)	Water ($n = 17$)	Sediment ($n = 5$)	Plankton ($n = 1$)
Ampicillin (10)	14 (77.8)	2 (11.1)	1 (5.6)	7 (30.4)	2 (8.6)	0 (0)
Chloramphenicol (30)	2 (11.1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ciprofloxacin (5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Furazolidone (100)	12 (66.7)	1 (5.6)	2 (11.1)	9 (39.1)	2 (8.6)	1 (4.3)
Gentamicin (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Nalidixic acid (30)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Nitrofurantoin (300)	7 (38.9)	2 (11.1)	1 (5.6)	0 (0)	0 (0)	0 (0)
Streptomycin (10)	13 (72.2)	2 (11.1)	2 (11.1)	2 (8.6)	1 (4.3)	1 (4.3)
Tetracycline (30)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

nate with the O139 or with the O1 antisera (non-O1 non-O139) produced CT. The frequency of resistance to various antibiotics of strains of *V. cholerae* O139 was compared with that of *V. cholerae* non-O1 non-O139 strains (Table 2). Resistance to ampicillin, furazolidone and streptomycin was encountered among strains belonging to both *V. cholerae* O139 and *V. cholerae* non-O1 non-O139 strains, but the percentage of resistant strains in the former was much higher than in the latter. Further, all the O139 strains examined were resistant to pteridine (both 10 and 150 μg) while only 56.5% of the strains in the non-O1 non-O139 group were resistant to pteridine. In all, a total of 7 resistance patterns were encountered among the O139 serogroup with strains showing the pattern AM,FZ,NF,ST (38.8%) being the most common while among the non-O1 non O139 serogroups, 9 R patterns were encountered.

Discussion

The explosive epidemic of cholera caused by the O139 serogroup of *V. cholerae* in Calcutta provided us with a unique opportunity to assess the role of aquatic water bodies in cholera endemic areas during the period of the epidemic. An ecological study on *V. cholerae* conducted in the same aquatic water body between July 1984 and June 1985 [17] when there was no epidemic of cholera also afforded us an opportunity to compare the situation of *V. cholerae* in an aquatic realm during, and in the absence of, an epidemic of cholera.

The highest isolation of *V. cholerae* O139 in this study was made from surface waters indicating the gross contamination of the freshwater lake during the period of the O139 cholera epidemic in Calcutta. Although the lake water is not used for drinking purposes, people residing in the vicinity of this lake utilize it for a variety of other purposes such as washing clothes and utensils and for ablution and bathing. Epidemiological studies on cholera have clearly documented that persons who used culture-positive water sources for cooking, bathing or washing, but culture-negative sources for drinking had the same rate of infection as persons who used culture-positive water for drinking [24]. This would indicate that the lake waters substantially contributed to the dissemination of O139 cholera during the epidemic.

The higher density of the O139 serogroup in surface waters as compared to sediment and plankton samples point towards an allochthonous source of the O139 serogroup probably from ambulatory cases or carriers. The isolation of the O139 serogroup was higher during the epidemic period and steadily declined thereafter. Several ecological factors such as competition with other organisms for nutrients, exposure to sun light and/or transformation of the O139 serogroup into a non-culturable but viable state [6], could have contributed to the rapid decline of the O139 serogroup with the weakening of the epidemic. It has similarly been documented that even in heavily contaminated waters *V. cholerae* O1 was found to disappear when the human source was removed [25]. It appears from this and from previous studies [25] that for some, hitherto unexplainable, reason the resident *V. cholerae* non-O1 non-O139 have a competitive edge over the toxigenic O1 and O139 serogroups of *V. cholerae* in aquatic environments resulting in the rapid depletion of the densities of the latter on the decline of an epidemic or on the removal of the contaminating human source.

All the strains of *V. cholerae* belonging to the O139 serogroup recovered from the environmental samples in this study were toxigenic. The more interesting finding, however, was that a substantial percentage of the non-O1 non-O139 *V. cholerae* strains were also toxigenic. This is in sharp contrast to the data obtained when we conducted the environmental study in the same area in the absence of an epidemic. Of the 371 strains of *V. cholerae* non-O1 isolated between July 1984 and June 1985 from different samples of the same freshwater lake, only 2 (0.5%) produced CT [26]. In contrast, 7.9% of the non-O1 non-O139 strains isolated in this study produced CT indicating a 16-fold increase in the toxigenicity of the resident *V. cholerae* non-O1 non-O139 isolates. A hypothesis would be that during a

cholera epidemic due to the introduction of large numbers of *V. cholerae* like the O139 serogroup, in this instance, there is an increase in the numbers of toxigenic vibrios among the innocuous aquatic residents facilitated by means of genetic exchange with some potential to provide a reservoir to reinitiate an epidemic.

Although the drug resistance patterns between strains of *V. cholerae* belonging to the O139 and the non-O1 non-O139 serogroups were more or less similar, more number of strains belonging to the O139 serogroup were resistant to ampicillin, furazolidone, streptomycin and to the vibriostatic agent O/129. This pattern of resistance was more or less similar to the resistance pattern exhibited by *V. cholerae* belonging to the O1 serogroup isolated from cholera patients from the Infectious Diseases Hospital for the past two years [27,28] before the O139 serogroup came into being.

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