APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1985, p. 132–136
0099-2240/85/010132-05$02.00/0
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Vol. 49, No. 1

Seasonal Distribution of *Vibrio parahaemolyticus* in Freshwater Environs and in Association with Freshwater Fishes in Calcutta

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Received 12 July 1984/Accepted 17 October 1984

The seasonal distribution of *Vibrio parahaemolyticus* in freshwater environs and in association with freshwater fishes was studied in 1982 and 1983. The occurrence of this organism in water and sediments at the three sites studied was very infrequent and was restricted to the summer months, although it was not always isolated during these months. The association of *V. parahaemolyticus* with plankton was chiefly confined to the summer months and progressively declined with the onset of monsoons, remaining below detectable levels during the postmonsoon and winter months. The incidence and counts of *V. parahaemolyticus* were consistently higher in association with plankton than with water and sediment samples. *V. parahaemolyticus* could be recovered throughout the period of investigation from freshly caught and market samples of freshwater fishes. The highest recovery rate of this halophile from fishes was invariably from fecal samples. Most of the strains isolated in this study were untypable, and those which could be typed were predominantly serotypes encountered in the environment. All the isolates were Kanagawa negative. From this study, it could be concluded that the survival of *V. parahaemolyticus* in freshwater ecosystems is transient and dependent on a biological host.

Although the ecology of *Vibrio parahaemolyticus* has been thoroughly studied in temperate (7) and tropical (G. B. Nair, Ph.D. thesis, Annamalai University, India, 1982) estuarine environs, one aspect which remains unexplored is its freshwater distribution. The occurrence of this moderately halophilic organism in fresh water has been construed as a fortuitous incidence that is probably related to tidal drift of the organism from the upper reaches of rivers (9) or to its introduction by ambulatory cases or carriers (16). The survival of *V. parahaemolyticus* in freshwater systems has remained inexplicable, since it is a halophilic organism.

Calcutta, located in the gangetic plains of India, is an endemic area for diarrheal diseases. Etiological studies on acute diarrheal diseases in this area have shown that gastroenteritis caused by *V. parahaemolyticus* ranks second to cholera in terms of incidence (2, 15). Epidemiological studies have further revealed the high incidence of human carriers of *V. parahaemolyticus* in this metropolis (4). The halophile has also been isolated from environmental samples, particularly from crabs and finfish (3). In an earlier study, Sarkar et al. (16) demonstrated the association of *V. parahaemolyticus* with plankton harvested from freshwater areas in Calcutta. However, apart from reporting the incidence of this halophile in freshwater areas, no previous studies have attempted to investigate the seasonal distribution or explain the natural habitat of this organism, if any, in freshwater systems. This prompted us to initiate a comprehensive investigation of the seasonal variations of *V. parahaemolyticus* in natural freshwater environs of Calcutta.

MATERIALS AND METHODS

Sampling sites. Three sampling stations (Fig. 1) located in various parts of Calcutta (longitude, 88°20′ E; latitude, 22°32′ N) and representative of different aquatic bodies were sampled once a month from March 1982 through February 1983. Stations 1 and 2 were totally freshwater locales and had no confluence whatsoever with marine or brackish waters. Station 1 was a man-made lake occupying 39.5 acres, with an essentially subterranean source, whereas station 2 was a tank receiving treated sewage water and is used for culturing freshwater fishes. A sampling point ca. 80 miles inshore from the Bay of Bengal in the river Hooghly was selected as station 3. There is a slight influx of seawater during the summer months at this station. On the basis of hydrographical events which are chiefly influenced by the monsoons, the study period could be demarcated into four seasons, summer (March to June), monsoon (July to September), postmonsoon (October to November), and winter (December to February).

Collection of environmental samples. Planktonic organisms were harvested by towing a horizontal plankton net (bolting silk no. 20; mesh size, 77 μm) for ca. 30 min at a depth of ca. 0.5 m below the surface and then transferred into sterile wide-mouth glass bottles. Presterilized glass bottles were used to collect water samples just below the water surface (ca. 0.5 m). A Petersen Grab, which was thoroughly rinsed and air dried before use, was used to collect bottom sediments.

Physical and chemical parameters. Methods used for the measurement of physical and chemical parameters, i.e., temperature, salinity, dissolved oxygen, turbidity, and pH, have been previously reported (13).

Bacteriological methods. Total viable aerobic heterotrophic counts (TVC) were determined on nutrient agar (Difco Laboratories) without added sodium chloride by the spread plate technique. The methodology used for enumeration of *V. parahaemolyticus* was the three-tube most-probable-number procedure described by Kaneko and Colwell (7), incorporating recent modifications developed at the University of Maryland (J. B. Kaper, Ph.D. thesis, Georgetown University, Washington, D.C., 1979). Appropriate decimal dilutions of homogenized plankton, water, and sediment were serially prepared by using the sterilized water sample from the collection area as diluent. A modified

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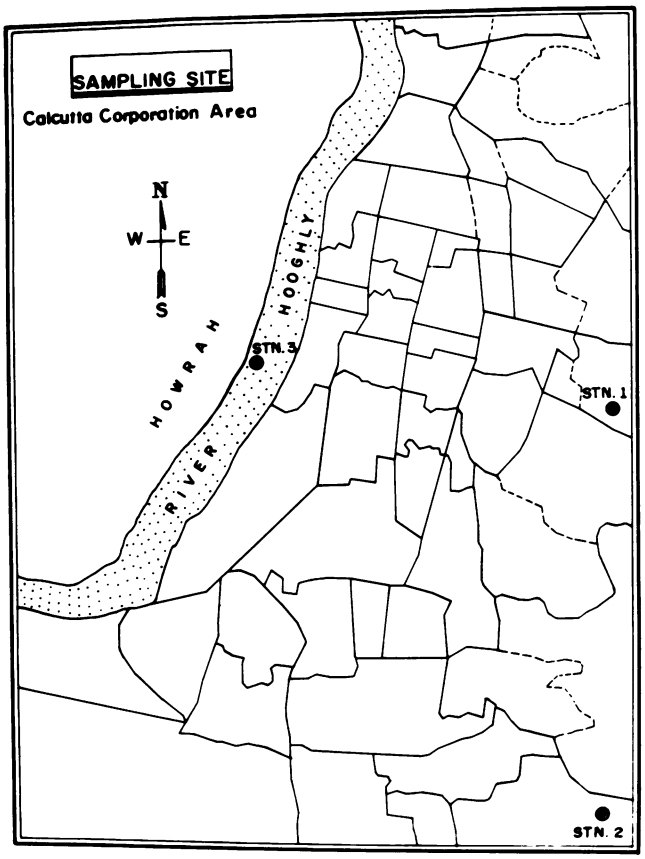

FIG. 1. Location of stations sampled in the Calcutta Corporation area.

arabinose ethyl violet broth was used as the enrichment medium. Larger volumes of the water sample were filtered through 0.45-μm filters, which were then placed into the above enrichment broth. After incubation for 24 h at 37°C, the enrichment broth cultures were plated on thiosulfate citrate bile salts sucrose agar (Eiken) and incubated for 24 h at 37°C. Colonies were picked, and the isolates were presumptively identified by using the multitest screening medium (8). The presumptive identifications were confirmed by additional tests advocated by Sakazaki (14). Most-probable-number values of samples yielding confirmed isolates of *V. parahaemolyticus* were determined from published tables (1).

Fish samples. Fishes sampled were of two categories, i.e., freshly caught and market samples of fishes. This was done to determine whether *V. parahaemolyticus* was associated with fishes in the environment or was a result of secondary contamination during the marketing process. Freshly caught fishes were harvested from station 2 by using indigenous gears and sampled within 2 h in the laboratory. This survey was conducted for 5 months (October 1982 through February 1983). Market samples of fishes were randomly purchased from a number of vendors at different markets for 1 year (March 1982 through February 1983). Fishes from both these sources comprised common edible freshwater species which form a staple diet of the local inhabitants. At the laboratory, sand and detritus adhering to the specimens were washed off with sterile saline. In all specimens, swabs of the external surface, gills, and fecal matter were taken as described by Nair et al. (11). All swabs were inoculated into arabinose ethyl violet broth and incubated for 24 h at 37°C. Subsequently, two loopfuls of the broth were plated on thiosulfate citrate bile salts sucrose agar and incubated for 24 h at 37°C. Colonies were picked and identified as described above.

Kanagawa phenomenon and serotyping. Determination of Kanagawa phenomenon and serotyping of the confirmed strains were performed by established procedures (14). Strains which showed partial or indeterminate hemolysis on Wagatsuma agar were further tested for Kanagawa activity by the modified Elek test (5).

RESULTS

The range of variations in environmental parameters and in TVC monitored in this study at the three stations are given in Table 1. Surface water temperatures at all stations varied between 19 and 34°C, with higher temperatures recorded during the summer months. Salinity at all three stations was found to be uniformly low (<1.6‰), with no major seasonal fluctuation. Relatively higher salinity was observed at station 3 and could be related to the slight seawater influx during the summer months. Variations in turbidity, pH, dissolved oxygen, and TVC did not fall into readily discernible seasonal patterns.

Seasonal incidence and counts of *V. parahaemolyticus* in plankton, water, and sediments at the three stations during the entire period of investigation are presented in Table 2. Among the three categories, the incidence and counts of *V. parahaemolyticus* were consistently higher in association with plankton. *V. parahaemolyticus* was recovered from

TABLE 1. Range of variations in environmental parameters and TVC at the three stations during the period of investigation

Station no.	Source of sample	Temp (°C)	Salinity (‰)	Turbidity (*K*)	pH	Dissolved O_2 (ml/liter)	TVC (CFU/ml) ($\times 10^4$)
1	Plankton	—[a]	—	—	—	—	13–530
	Water	24–34	0.04–1.6	0.1–3.4	6.0–8.4	8.5–14.6	0.09–18
	Sediment	23–32	—	—	6.5–8.8	—	1–270
2	Plankton	—	—	—	—	—	27–9,700
	Water	20–33	0.01–0.5	0.1–18.8	6.0–8.8	8.1–14.7	0.18–55
	Sediment	19–32	—	—	6.4–8.8	—	4.7–810
3	Plankton	—	—	—	—	—	2.2–72
	Water	22–32	0.08–0.9	7.3–56.6	6.1–8.0	4.6–15.3	0.03–0.51
	Sediment	20–31	—	—	6.6–8.3	—	0.27–61

[a] —, Not determined.

TABLE 2. Seasonal incidence and counts[a] of *V. parahaemolyticus* measured between March 1982 and February 1983 in plankton, water, and sediments at the three stations

Station no.	Source of sample	Most-probable-number counts											
		March	April	May	June	July	August	September	October	November	December	January	February
1	Plankton	—[b]	40	7	90	0.4	—	43	15	—	—	—	—
	Water	0.3	0.4	0.9	0.9	—	—	—	—	—	—	—	—
	Sediment	0.3	—	—	0.4	—	—	—	—	—	—	—	—
2	Plankton	14	15	70	15	—	23	15	—	—	—	—	—
	Water	0.3	—	—	—	0.4	—	—	—	—	—	—	—
	Sediment	0.4	—	—	—	—	—	—	—	—	—	—	—
3	Plankton	—	—	4	—	—	—	0.4	—	—	—	—	—
	Water	—	—	—	—	—	—	—	—	—	—	—	—
	Sediment	0.3	0.3	—	—	—	—	—	—	—	—	—	—

[a] Most-probable-number counts expressed per gram (wet weight) for plankton and per 100 ml for water and sediment.
[b] —, Not detected.

plankton, water, and sediments mainly during the summer months, and the incidence progressively declined with the onset of monsoons and remained below detectable levels during the postmonsoon and winter months. *V. parahaemolyticus* could be detected more frequently in higher densities from station 1 than from stations 2 and 3.

The monthly incidence and occurrence of the halophile in the three areas (external surface, gills, and feces) of freshly caught freshwater fishes examined from station 2 is presented in Table 3. The halophile was found in 24 to 30% of the freshly harvested fishes over the period studied. The detection rate of *V. parahaemolyticus* among the three areas examined in the positive samples was highest in fecal samples (82.1%), whereas the frequency of isolation from the external surface was lowest (25%). The monthly incidence of *V. parahaemolyticus* in market samples of fishes is shown in Table 4. An exceptionally high overall isolation of 67.2% was observed. The isolation rate of *V. parahaemolyticus* in market samples of fishes ranged from 15 to 90%, with no apparent seasonal fluctuations. As observed in freshly caught fishes, the organism was found most frequently in the fecal samples of market fishes.

All the strains isolated in this study were Kanagawa phenomenon negative. The distribution and frequency of occurrence of serotypes of *V. parahaemolyticus* from different categories of samples (plankton, water, sediment, and fishes) are presented in Table 5. Of the 93 strains examined, 27 (29%) could be typed as belonging to a currently acknowledged serotype of *V. parahaemolyticus*. The number of serotypes recorded in this study included 11 individual serotypes, which represents 16.2% of the possible 74 O-K combinations described in the antigenic schema of *V. parahaemolyticus*. One atypical serotype, O2:K17, which is not listed by the Japanese committee on the serological typing of *V. parahaemolyticus*, was isolated from a plankton sample.

DISCUSSION

By integrating the data obtained on the occurrence of *V. parahaemolyticus* in plankton, water, and sediment samples and in freshly caught and market samples of fishes, a fairly clear idea on the occurrence and distribution of this organism in freshwater environs of Calcutta emerged. The incidence of *V. parahaemolyticus* strains in freshwater ecosystems is primarily related to their association with a biological host, particularly fishes.

The occurrence of *V. parahaemolyticus* in water and sediments at the three stations was very infrequent and random. Most of the isolations of this organism occurred during the summer months, although it was not always isolated during these months. Even when optimum temperatures prevailed, *V. parahaemolyticus* in most instances was not detected in the water column or in the sediments at the three stations. The low salinity of freshwaters and the relatively oligotrophic nature of these niches are probably the factors limiting the distribution of this moderately halophilic organism in freshwater and freshwater sediments.

TABLE 3. Incidence of *V. parahaemolyticus* in freshly caught freshwater fishes from station 2

Date	No. examined	No. (%) positive for *V. parahaemolyticus*	No. (%) positive for *V. parahaemolyticus* in three regions[a]:		
			External suface	Gills	Feces
1982					
October	20	6 (30.0)	3 (50.0)	4 (66.6)	6 (100.0)
November	25	7 (28.0)	1 (14.3)	2 (28.5)	4 (57.1)
December	20	5 (25.0)	1 (20.0)	3 (60.0)	5 (100.0)
1983					
January	15	4 (26.6)	0	2 (50.0)	3 (75.0)
February	25	6 (24.0)	2 (33.3)	2 (33.3)	4 (66.6)
Total	105	28 (26.6)	7 (25.0)	13 (46.4)	23 (82.1)

[a] Number of positive isolations from respective region per number of fishes positive for *V. parahaemolyticus*.

TABLE 4. Incidence of *V. parahaemolyticus* in samples of freshwater fishes obtained from markets

Date	No. examined	No. (%) positive for *V. parahaemolyticus*	No. (%) positive for *V. parahaemolyticus* in three regions[a]:		
			External surface	Gills	Feces
1982					
March	20	10 (50.0)	5 (50.0)	4 (40.0)	7 (70.0)
April	10	9 (90.0)	6 (66.6)	5 (55.5)	6 (66.6)
May	20	3 (15.0)	0	1 (33.3)	3 (100.0)
June	25	16 (64.0)	4 (25.0)	6 (37.5)	8 (50.0)
July	15	11 (73.3)	0	2 (27.2)	10 (90.9)
August	20	14 (70.0)	2 (14.2)	7 (50.0)	8 (57.1)
September	25	20 (80.0)	3 (15.0)	11 (55.0)	15 (75.0)
October	25	21 (84.0)	6 (28.5)	8 (38.0)	17 (80.9)
November	30	26 (86.6)	9 (34.6)	9 (34.6)	22 (84.6)
December	20	10 (50.0)	3 (30.0)	9 (90.0)	10 (100.0)
1983					
January	20	14 (70.0)	4 (28.5)	8 (57.1)	8 (57.1)
February	20	14 (70.0)	3 (21.4)	7 (50.0)	11 (78.5)
Total	250	168 (67.2)	45 (26.7)	78 (46.4)	125 (74.4)

[a] Number of positive isolations from respective region per number of fishes positive for *V. parahaemolyticus*.

Plankton in freshwater environs appears to aid the survival of *V. parahaemolyticus* by the process of adsorption onto the planktonic substrate. This, however, is temperature related, occurring largely during the summer months. In the postmonsoon and winter months, *V. parahaemolyticus* could not be isolated from plankton, water, or sediment samples at the three stations. During these months, although there were no major changes in the salinity profile (apart from a slight decline), *V. parahaemolyticus* remained below detectable levels. Possibly the decline in water temperature excludes even a transient survival of *V. parahaemolyticus* in the freshwater environment. Obviously, during these months the two important factors, i.e., salinity and temperature, are suboptimal, resulting in the virtual elimination of the halophile from the environment. During summer months, despite extremely low salinity, water temperature is within the optimal limits, which perhaps aids the survival of the organism in the environment, particularly in association with plankton.

TABLE 5. Distribution and frequency of occurrence of serotypes of *V. parahaemolyticus* from different categories of samples

Serotype	Sample category				Total $n = 93$
	Plankton ($n = 25$)	Water ($n = 10$)	Sediment ($n = 7$)	Fishes ($n = 51$)	
O1:K1		1			1
O2:K3			1	2	3
O2:K17*a*	1				1
O2:K28	2	3	1	1	7
O3:K6	1				1
O3:K29				1	1
O4:K10			1		1
O4:K34				1	1
O4:K42				2	2
O4:K67				1	1
O5:K15	1		1	1	3
O5:K17				5	5
Typable	5 (20.0)[b]	4 (40.0)	4 (57.1)	14 (27.4)	27 (29.0)
Untypable	20 (80.0)	6 (60.0)	3 (42.8)	37 (72.5)	66 (71.0)

[a] Atypical serotype which does not fall into the serotypes listed by the Japanese committee on the serological typing of *V. parahaemolyticus*.
[b] Numbers in parenthesis indicate percentage.

Therefore, adsorption onto plankton could be viewed as a process which prolongs the survival of *V. parahaemolyticus* in the freshwater environment by conferring some kind of hitherto unknown protection. Recent studies on the attachment of *V. cholerae* to copepods have indicated the possibility that live copepods may excrete growth-promoting or chemical attractant compounds which might enhance the attachment of chitinolytic vibrios to copepods (6). Furthermore, the association of *V. cholerae* with chitin has been found to confer resistance to acid pH on this organism (12).

Cells of *V. parahaemolyticus* are likely to be released during the disintegration of the planktonic substrate but apparently do not survive in water or sediment in freshwater areas, as reflected by the total absence or low counts encountered in this study. From this study, it is obvious that the two links necessary to complete a cycle in the freshwater system, i.e., water and sediments, do not sustain *V. parahaemolyticus*. Therefore, despite the presence of *V. parahaemolyticus* in association with plankton and fishes in freshwater environs, no kind of a flux of cells within or between heterotrophic niches could be conceivable.

This study, however, provides conclusive evidence that, irrespective of their origin (marine or freshwater), fishes provide an ideal substrate for the survival and proliferation of *V. parahaemolyticus*. Fishes, and perhaps other aquatic animals, are reservoirs of *V. parahaemolyticus* in freshwater environs. The highest recovery rates of this halophile in both freshly caught and market samples of freshwater fishes were from the fecal swabs, suggesting that the gastrointestinal tracts of freshwater fishes provide a unique microcosm for the proliferation of *V. parahaemolyticus* in freshwater areas. Consequently, fecal matter of fishes could form a significant source of inoculum of the halophile into the surrounding environment. The survival of such cells entering into the environment is likely to be transient and to be dependent on another host and on the prevailing environmental conditions.

The higher isolation rates of *V. parahaemolyticus* in the market samples of fishes than in the freshly caught fishes could be attributed to cross-contamination due to mishandling at the fishmongers' stalls. Exposure to ideal ambient temperatures could be another factor contributing to the higher incidence.

In an earlier study, it was thought that the occurrence of *V. parahaemolyticus* in freshwater environs in Calcutta may be related to the introduction of cells by ambulatory cases or human carriers (16). Data emerging from serotyping of the strains isolated in this study, however, do not support the above hypothesis. Most of the strains were untypable, and those which could be typed were predominantly serotypes normally encountered in environmental samples. Also, all the strains were Kanagawa phenomenon negative. Serotypes generally associated with human disease in Calcutta were conspicuous by their absence. A synonymous pattern of distribution of *V. cholerae* in natural environs exists, with the epidemic O1 serotype being sparsely distributed in natural environs, whereas the non-O1 serovars are abundantly found in the aquatic system (10).

The association of *V. parahaemolyticus* with freshwater fishes and their ability to survive in association with freshwater plankton under certain environmental conditions is, indeed, significant. Recent studies on the Na^+ requirement of *V. parahaemolyticus* and *V. cholerae* have indicated that, in contrast with other marine bacteria, the quantitative requirements for Na^+ for growth vary with the substrate serving as the carbon and energy source in the medium (R. A. MacLeod, personal communication). This would imply that, under certain specific nutrient conditions, the Na^+ requirement of *V. parahaemolyticus* is not mandatory and that the halophile can well survive in conditions where the salt concentration may be equal to or even lower than physiological concentrations. An important issue now would be whether *V. parahaemolyticus* is a truely marine organism or whether it belongs to an as yet undefined "twilight" classification.

ACKNOWLEDGMENTS

We thank S. P. De and B. K. Sircar, Assistant Directors of the National Institute of Cholera and Enteric Diseases, for their assistance during the major part of this study. Y. Takeda, Research Institute for Microbial Diseases, Osaka University, Japan, kindly supplied filter strips impregnated with antihemolysin serum.

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