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Ecology, Serology, and Enterotoxin Production of *Vibrio cholerae* in Chesapeake Bay

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A total of 65 isolates of *Vibrio cholerae*, serotypes other than O-1, have been recovered from water, sediment, and shellfish samples from the Chesapeake Bay. Isolations were not random, but followed a distinct pattern in which salinity appeared to be a controlling factor in *V. cholerae* distribution. Water salinity at stations yielding *V. cholerae* (13 out of 21 stations) was 4 to 17‰, whereas the salinity of water at stations from which *V. cholerae* organisms were not isolated was <4 or >17‰. From results of statistical analyses, no correlation between incidence of fecal coliforms and *V. cholerae* could be detected, whereas incidence of *Salmonella* species, measured concurrently, was clearly correlated with fecal coliforms, with *Salmonella* isolated only in areas of high fecal coliform levels. A seasonal cycle could not be determined since strains of *V. cholerae* were detectable at low levels (ca. 1 to 10 cells/liter) throughout the year. Although none of the Chesapeake Bay isolates was agglutinable in *V. cholerae* O group 1 antiserum, the majority were toxigenic; i.e., approximately 87% of the isolates exhibited toxicity for Y-1 adrenal cells. Furthermore, rabbit ileal loop and mouse lethality tests were also positive for the Chesapeake Bay isolates, with average fluid accumulation in positive ileal loops ranging from 0.21 to 2.11 ml/cm. Serotypes of the strains of *V. cholerae* recovered from Chesapeake Bay were those of wide geographic distribution. It is concluded from the data assembled to date, that *V. cholerae* is an autochthonous estuarine bacterial species resident in Chesapeake Bay.

The occurrence of *Vibrio cholerae* in areas where cholera is not endemic has rarely been reported. Outbreaks of cholera in the United States have virtually been eliminated, with only two cases of nonimported, nonlaboratory-acquired cholera being reported in the United States since 1911 (5, 46). The recent isolation of toxigenic strains of *V. cholerae* from the Chesapeake Bay, the largest estuary on the east coast of the United States, is of great interest for microbial ecology, as well as epidemiology, since strains of *V. cholerae* were isolated from both polluted and unpolluted areas of the Chesapeake Bay (12). Chesapeake Bay strains were capable of producing enterotoxin, as demonstrated by rabbit ileal loop (RIL) and Y-1 adrenal cell assays.

The definition of *V. cholerae* provided by Koch in 1882 (25) has been broadened in accordance with improved understanding of the taxonomy and pathogenicity of *V. cholerae* so that the genospecies and taxospecies of *V. cholerae* now include "*Vibrio* El Tor." The El Tor vibrios differ from classical *V. cholerae* in hemolysin production and other properties but for many years were not associated with cholera per se or even considered to be a pathogen of significance. In recent years, the El Tor vibrios have come to be recognized as a biotype of *V. cholerae* and are recognized, along with classical *V. cholerae*, as epidemiological agents. The genospecies and taxospecies of *V. cholerae* also include strains which do not possess the group O-1 somatic antigen, formerly referred to either as non-agglutinable or noncholera vibrios, both of which are misnomers since so-called non-agglutinable vibrios will readily agglutinate in antisera prepared for these strains, and the term "non-cholera vibrio", can be applied to *V. parahaemolyticus, V. anguillarum,* or other *Vibrio* species. Data obtained from numerical taxonomy, isozymic analysis, DNA base composition, and DNA/DNA hybridization analyses indicate that the so-called "non-agglutinable" strains of *Vibrio* belong within the taxospecies and genospecies of *V. cholerae* (8, 9, 22, 36, 38, 45). Thus, the only taxonomic criterion distinguishing *V. cholerae* from so-called non-agglutinable *V. cholerae* is the lack of an antigen. Agglutinable strains of *V. cholerae* have been reported to lose

the group 1 antigen and non-agglutinable strains of *V. cholerae* to gain ability to agglutinate in cholera group 1 antiserum (3, 18, 41). Pathogenicity of serotypes of *V. cholerae,* other than O-1, has recently been recognized, and several reports of outbreaks of cholera-like disease associated with such strains include symptoms ranging from mild diarrhea to severe cholera. Aside from repeated isolations of *V. cholerae,* other than O-1, from cases of diarrhea (23, 27, 30, 47), these serotypes of *V. cholerae* have also been implicated in septicemia and meningoencephalitis (17, 23, 33, 34).

Laboratory studies with strains of *V. cholerae,* other than O-1, employing RIL and other tests for enteropathogenicity, have demonstrated that these strains can be considered potential pathogens (16, 31, 48). From results of an extensive taxonomic study of the so-called non-agglutinable vibrios, Sakazaki et al. concluded that all of the strains tested produced diarrhea similar to that caused by the cholera vibrio (36). Zinnaka and Carpenter (48) and Ohashi et al. (31) studied enterotoxin produced by strains of *V. cholerae* of serotype other than O-1 and concluded that the toxin was closely related to enterotoxin produced by the classical *V. cholerae* strain 569B.

In general, the ecology of *V. cholerae* is not well understood, and, unless associated with disease in a nearby community, little significance has been attached to the occurrence of non-O-1 serotype *V. cholerae.* The studies that have been reported (29) are often marred by poor identification procedures, employing too few tests for separation of *V. cholerae* from *Aeromonas, Pseudomonas,* and related organisms. Such identifications are all too frequent in the literature and are responsible, in a large part, for the failure of many epidemiologists to view the occurrence of serotypes of *V. cholerae* other than O-1 as a public health hazard. Because cholera is not widespread in North America, *V. cholerae* is assumed to be absent from North American waters, an unwarranted assumption.

The objective of the studies reported here was to determine the incidence of *V. cholerae* in the Chesapeake Bay. Subsequent to our discovery of *V. cholerae* in Chesapeake Bay (12), an extensive survey of the Chesapeake Bay and its tributaries was undertaken, with the additional isolates also tested for enterotoxin production. Thus, we report results of studies on the ecology, distribution, serology, and potential pathogenicity of *V. cholerae* in Chesapeake Bay.

MATERIALS AND METHODS

Sampling sites. A total of 21 stations throughout the Chesapeake Bay was sampled between October 1976 and January 1978, including stations shown in Fig. 1. It should be noted that stations 1, 2, 3, 12, 13, 14, 15, 16, and 17 are approximately 40 to 50 km apart along the entire length of the Bay, encompassing a significant salinity gradient. Stations 18, 19, 20, and 21 are located in the James River, a major tributary of Chesapeake Bay, which also provides a salinity gradient. Station 11, Colgate Creek (see Fig. 1), is in Baltimore Harbor, an area severely polluted by both domestic and industrial wastes. A study undertaken in July 1977 comprised a transect from the inner Baltimore Harbor, the most heavily polluted site in Chesapeake Bay, to the Chester River, which is relatively unpolluted. Thus, stations 3 to 10 (Fig. 2) provided a "pollution gradient" with respect to the sampling regimen.

Collection of samples. Water samples were collected with a 2-liter sterile Niskin Bag sampler (General Oceanics, Miami, Fla.) or, for larger volumes, with a submersible pump, which was thoroughly flushed with water from the site to be sampled before sample collection. Water samples were taken, except where otherwise noted, 1 to 2 m below the surface and were briefly held, after collection, in the Niskin Bag or in sterile Nalgene carboys, with all samples processed aboard ship immediately after collection.

The upper 10 cm of sediment was sampled using a nonaseptic Petite Ponar grab sampler (Wildlife Supply Co., Saginaw, Mich.). Sediment samples were subsampled aseptically for microbiological examination. Oysters were collected by dredge and aseptically shucked, and their tissues were excised and homogenized with 100 ml of 0.5% peptone broth, yielding a final dilution of 1:3.

Physical and chemical parameters. Dissolved oxygen was determined by titration, using a modified Winkler method (6). Temperature and salinity were measured by the Induction, Conductivity, Temperature, Indicator unit available aboard the R/V *Ridgely Warfield.* Transparency was derived from Secchi disk readings, whereas total suspended matter was determined according to standard methods (1).

Bacteriological analyses. Total viable aerobic heterotrophic counts were determined by using upper Bay yeast extract agar (37). Salinity of the medium was increased for lower Bay stations to correspond to in situ salinity (15 to 20‰).

Presumptive total and fecal coliforms were estimated by using lactose and EC broths (Difco Laboratories, Detroit, Mich.), respectively, in a three-tube replication of a most-probable-number (MPN) series (1).

Salmonella spp. were isolated by a primary, nonselective enrichment technique found to be extremely useful in earlier studies carried out in the Chesapeake Bay (24). Estimates of *Salmonella* populations were determined by a three-dilution, three-tube replication series to derive the MPN.

V. parahaemolyticus were enumerated by an MPN procedure whereby 100-, 10-, and 1-ml sample volumes are inoculated into a modified arabinose, ethyl violet broth (21) containing (grams per liter): peptone (Difco), 5.0; beef extract (Difco), 3.0; NaCl, 30; bromothymol blue, 0.03; ethyl violet, 0.001; and galactose, 5.0 (pH 9.0). Double-strength broth was used for 10-

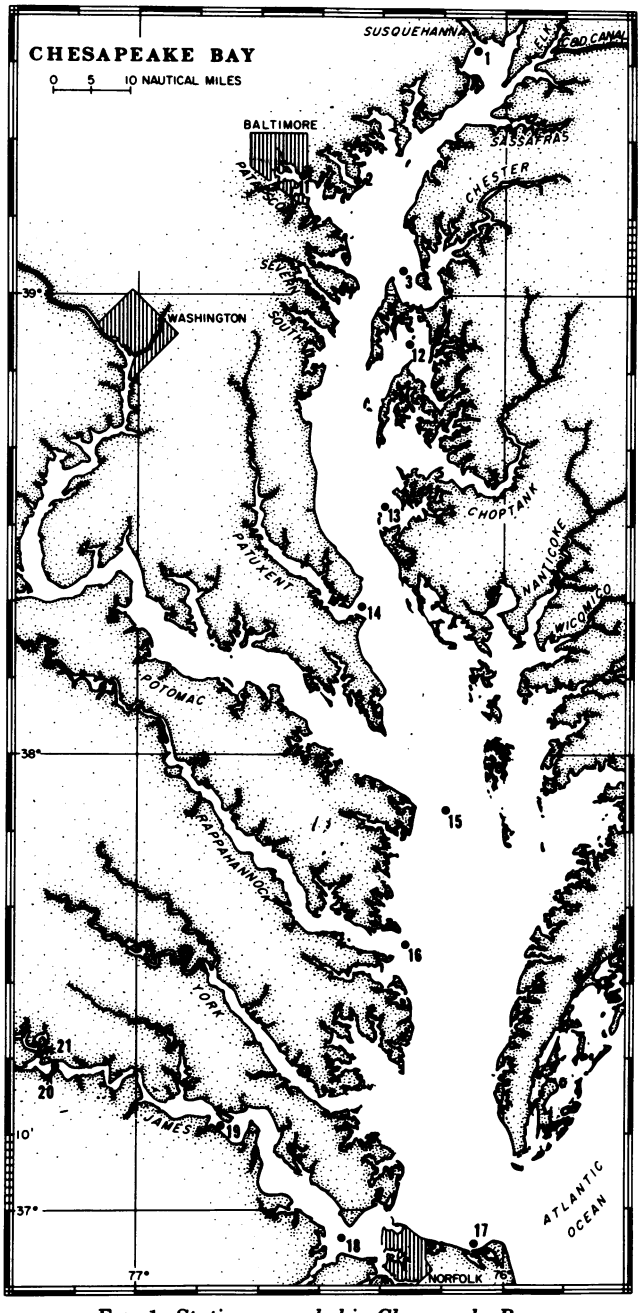

FIG. 1. *Stations sampled in Chesapeake Bay.*

ml volumes of sample, and single-strength broth for 1-ml and 100-ml volumes, with the 100-ml volume samples first being concentrated using 0.45-μm membrane filters. After incubation for 24 h at 37°C, the enrichment broth cultures were streaked onto thiosulfate citrate bile salts agar (TCBS) plates (Difco) which were incubated at 37°C for 24 h. Colonies were picked, purified, and characterized by a series of biochemical tests. Strains yielding the following reactions were recorded as presumptive *V. parahaemolyticus*: cytochrome oxidase (+), growth in 0% NaCl (−), growth in 3% NaCl (+), growth at 43°C (+), acid from glucose (+), acid from lactose (−), acid from sucrose (−), production of H_2S (−), arginine dihydrolase (−), and lysine decarboxylase (+).

Vibrio spp. were enumerated using the medium of

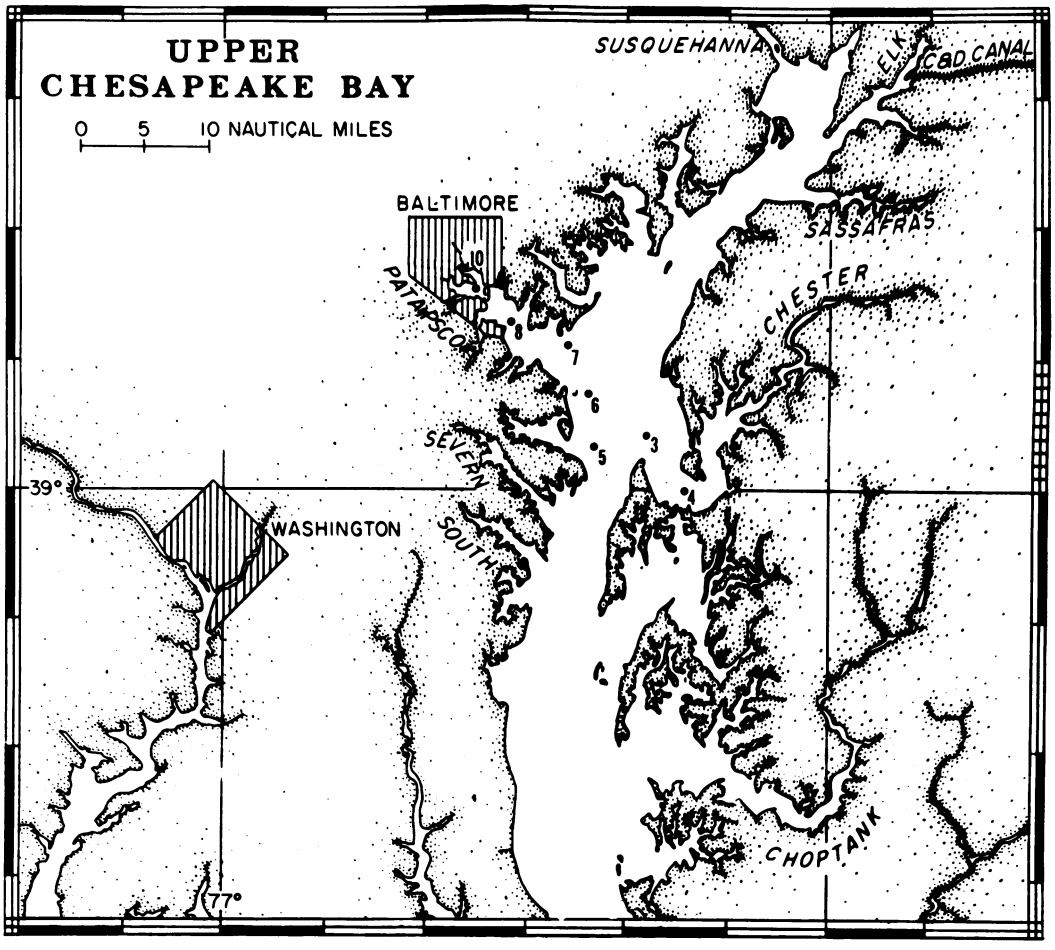

FIG. 2. *Stations sampled on transect cruise, July 1977.*

Simidu et al. (39).

Isolation of *V. cholerae*. *V. cholerae* were found to be present in very low numbers in Chesapeake Bay water and sediment samples. Thus, concentration by filtration was necessary for recovery of *V. cholerae* from the environment. Celite (Johns-Manville), a filtering adjuvant prepared in stock solution and autoclaved at 121°C (15 lb/in^2 pressure [10^5 N/m^2]) for 15 min was added to the water samples to a final concentration of 1 g/liter. After thorough agitation, 100- or 500-ml volumes of water were filtered through a 0.45-μm filter, after which the filters and Celite were placed in flasks containing 50 or 100 ml of enrichment broth. A filter through which 100 ml of sample was passed was placed into each of three bottles containing 50 ml of broth. For 1,000-ml sample volumes, two filters, through which had been passed 500 ml of sample, were placed into each of three bottles containing 100 ml of broth. The third dilution for the MPN series utilized 10 ml of sample volume inoculated into 10 ml of double-strength broth. The broth was a simple alkaline peptone enrichment broth containing peptone (Difco) (10 g/liter) and NaCl (10 g/liter) at pH 8.5.

After inoculation, the enrichment flasks were incubated for 18 h at 37°C, at which time they were streaked onto TCBS and incubated for 18 to 24 h at 37°C. If the TCBS plates yielded no *V. cholerae*, the enrichment broths were held at 10°C and restreaked approximately 1 week later. This procedure often led to recovery of isolates that had not been detected at the time of initial streaking.

Isolated colonies were screened for oxidase reaction. Oxidase-positive colonies were purified on tryptic soy agar (Difco) and further screened according to the following scheme: six to eight colonies were each streaked onto gelatin agar (40) containing no added NaCl. Strains growing on the 0% NaCl gelatin agar, that were gelatinase positive, were transferred to Kligler iron agar and to arginine dihydrolase and lysine decarboxylase media (Difco). Those strains that were oxidase (+), gelatinase (+), grew in medium with no added NaCl, were H_2S (−), K/A on Kligler iron agar, β-galactosidase (+), lysine (+), and arginine (−) were identified as presumptive *V. cholerae* and character-

ized further by additional biochemical tests, including the API 20 system (Analytab Products, Inc., Plainview, N.Y.). Isolates were sent to the Vibrio Reference Laboratory (VRL), Jefferson Medical College, Philadelphia, for confirmation and serotyping.

Statistical analyses. Physical, chemical, and bacteriological data were entered and stored on an IBM 370 computer. Multiple linear correlation coefficients were calculated by using the BMDP8D computer program of the Health Sciences Computing Facility, University of California, Los Angeles (14). Bacteriological data were transformed by a $\log_{10}$ transformation, and calculations were performed on the IBM 370 computer. Correlation coefficients (r) were tested for significance at the 95 and 99% confidence levels by comparing computed r values to tabulated critical r values, as given in Snedecor and Cochran (42).

Toxigenicity studies. The 65 strains of *V. cholerae*, characterized by the methods reported here and isolated from samples collected at the Chesapeake Bay stations, were maintained on Trypticase soy agar slants. Before examination for virulence, the strains were preconditioned by passage through 10 ml of brain heart infusion broth, followed by final static incubation in 25 ml of brain heart infusion medium in 150-ml screw-capped bottles.

Ligated RIL. The RIL tests were performed essentially as described by Spira and Goepfert (43); i.e., ca. 1.0 kg New Zealand white rabbits of either sex were anesthetized by intravenous injection of sodium pentobarbitol (45 mg/kg); the ileum was externalized and ligated, and 2.0 ml of either whole-culture or cell-free filtrate was injected into each loop. Test loops were separated by control intervals injected with sterile brain heart infusion broth. The ileum was returned, and the incision was closed. After 18 to 20 h, the rabbits were sacrificed and opened, and the loops examined and measured for fluid accumulation. Response was measured as the ratio of fluid accumulation to loop length in milliliters per centimeter. Most of the isolates were tested on three separate occasions.

Mouse intraperitoneal inoculations. For each strain, 0.5 ml of whole culture was inoculated intraperitoneally into two 18- to 21-day-old Swiss white mice, NIH/Nmri CV strain. The mice were retained in holding cages at room temperature and examined hourly for 10 h. Afterwards, examination was daily until day 10.

Y-1 mouse adrenal cell examination. Whole cultures were grown as described above and tested according to the method of Sack and Sack (35).

Serotyping of isolates. Cultures were typed by using live organisms, according to the slide agglutination test procedure described by Smith and Goodner (41).

RESULTS

Occurrence of *V. cholerae*. *V. cholerae* organisms were found to be ubiquitous in Chesapeake Bay. A total of 65 selected isolates were identified as *V. cholerae* on the basis of biochemical characterizations. None of the strains was agglutinable in *V. cholerae* O group 1 antiserum. In Table 1 are data concerning isolation of *V. cholerae* and physical, chemical, and bacteriological parameters of samples at the time of collection. The most striking pattern which emerged was that of the incidence of *V. cholerae* only within a given salinity range. The salinity of water at stations yielding *V. cholerae* was, without exception, 5 to 15‰.

A significant salinity gradient is a major characteristic of an estuary such as the Chesapeake

TABLE 1. *Isolation of V. cholerae in Chesapeake Bay*[a]

Station	Depth (m)	Transparency (m)	Temp (°C)	Salinity ‰	*V. cholerae* MPN[b]	TVC[c]	Coliform MPN[d]	Fecal coliform MPN[d]	Date
1	4.5	ND[e]	19.8	0.1	<0.3[f]	8.0×10^3	280	11	5/77
2	5.0	0.5	19.9	2.2	<0.3	8.8×10^4	110	1.7	5/77
3	11.9	2.0	20.0	10.1	7.0	1.6×10^3	ND	ND	6/77
11	10.4	1.0	21.8	7.6	0.9	2.8×10^6	ND	ND	6/77
12	8.2	2.0	28.8	11.5	0.9				7/77
13	5.5	2.9	21.6	11.9	1.1	1.3×10^3	ND	ND	6/77
14	15.0	2.8	24.7	14.6	1.5	2.6×10^3	23	4	6/77
15	13.0	2.3	24.9	14.5	0.4	4.4×10^3	<3	<3	6/77
16	11.6	1.7	24.3	20.7	<0.3	1.7×10^3	ND	ND	9/77
17	6.0	3.7	25.2	21.4	<0.3	2.1×10^3	150	150	6/77
18	10.0	1.5	25.7	19.1	<0.3	3.3×10^2	93	12	6/77
19	13.0	1.0	27.7	5.6	0.4	9.0×10^2	43	3	6/77
20	0.3	0.2	34.5	0.0	<0.3	2.5×10^7	46,000	46,000	6/77
21	7.5	1.0	27.5	0.2	<0.3	4.3×10^4	150	75	6/77

[a] Station numbers correspond to those in Fig. 1.
[b] *V. cholerae* MPN/1 liter of water.
[c] TVC, Total viable count/1 ml of water.
[d] MPN/100 ml of water.
[e] ND, Not determined.
[f] No *V. cholerae* isolated.

Bay. At the head of the Bay, the water can be considered freshwater due to the influx of the Susquehanna River into the upper Chesapeake Bay (Fig. 1). In the present study, *V. cholerae* organisms were not isolated at stations 1 and 2, the latter being located in the Back River, where the salinity of the water was 2.2‰. At the lower end of the Bay, the salinity of the water approached that of seawater. Thus, salinities at station 16, at the mouth of the Rappahannock, and station 17, at Little Creek, Va., were 20.7 and 21.4‰, respectively. *V. cholerae* were not isolated from water at the higher salinities, being limited to the middle range of salinities found in Chesapeake Bay. The distribution pattern was confirmed for the James River, a subestuary of the Chesapeake Bay, which also demonstrates an appreciable salinity gradient. At station 18, near Newport News, Va., where the salinity of the water was 19.1‰, no *V. cholerae* were isolated. Further up the James River, at station 19, near Jamestown, the salinity was 5.6‰, and the samples yielded *V. cholerae*. Stations 20 and 21, which can be considered freshwater, yielded no isolates.

No other physical, chemical, or microbiological parameters were observed to have a marked association with the incidence of *V. cholerae*. Interestingly, pH did not correlate with distribution, despite the well-recognized tolerance of *V. cholerae* to strongly alkaline pH (32); in fact, the largest numbers of *V. cholerae* were found at station 8 (Fig. 2), where the pH of the water was 6.4. To confirm the importance of salinity, a transect was made, following the dredged shipping channels from the Chester River, on the Eastern Shore of the Chesapeake Bay, across the Bay to the inner harbor of Baltimore. The transect comprised a significant pollution gradient, since Baltimore Harbor is one of the most heavily polluted areas of Chesapeake Bay, whereas the Chester River is relatively unpolluted (Fig. 2).

From Table 2 it can be seen that, except for salinity and temperature, a wide range of values for the physical, chemical, and microbiological parameters was obtained. Station 3, at the mouth of the Chester River, one of the least polluted stations, yielded dissolved oxygen values of 9.1 mg/liter and total viable counts of 1.6×10^4/ml, with no *Salmonella* or fecal coliforms isolated. *V. cholerae* were isolated at station 3, yielding an MPN of 0.7/liter. The MPN for *V. parahaemolyticus* was 9.3/100 ml, and the total *Vibrio* spp. count was 10/ml.

The other end of the pollution gradient, i.e., the inner Baltimore Harbor, as seen from data for stations 9 and 10, yielded very high numbers of fecal coliforms, 24,000/100 ml, and *Salmonella* spp., >240/100 ml. At station 9, which is near Fort McHenry, *V. cholerae* were isolated, yielding an MPN of 0.7/liter, similar to that for the Chester River. Station 10 is located at the confluence of Jones Falls and Baltimore Harbor. The dissolved oxygen content of the water was 0.1 mg/liter, i.e., essentially anoxic conditions. The pH of the water was 6.3, the lowest recorded for all samples. Bacterial numbers were extremely high, 1.1×10^7/ml with a total coliform MPN of >240,000/100 ml, a fecal coliform MPN of 46,000/100 ml, and a *Salmonella* MPN of 4.6/100 ml. *V. cholerae* organisms were not isolated, i.e., MPN of <0.3/liter.

Statistically significant correlations among the parameters with *V. cholerae* were sought. However, the bacterial numbers, after transfor-

TABLE 2. *Physical, chemical, and bacteriological data for Chesapeake Bay*[a]

Station	Transparency (m)	Temp (°C)	Salinity (‰)	DO[b] (mg/liter)	pH	Suspended particulates (mg/liter)	*Salmonella* MPN[c]	Coliform MPN[c]	Fecal coliform MPN[c]	TVC[d] (×10⁵)	Total *Vibrio* species	*V. parahaemolyticus* MPN[c]	*V. cholerae* MPN[e]
3	1.3	27.5	9.9	9.1	7.5	12.2	<0.03	24	<0.3	0.16	10	9.3	0.7
4	1.3	26.6	10.8	5.0	7.2	7.6	<0.03	46	4.3	0.1	14	24.0	1.5
5	1.0	29.4	8.2	12.5	8.8	12.4	<0.03	21	<0.3	0.6	27	1.5	0.4
6	1.2	28.2	7.8	10.3	8.0	7.8	<0.03	46	0.4	1.2	48	24.0	0.4
7	1.0	28.5	6.7	6.2	6.9	10.8	<0.03	150	<3	0.9	45	24.0	1.5
8	0.9	29.2	7.6	7.5	6.4	19.2	0.23	1,100	240	10.0	1,500	>240.0	46.0
9	0.9	27.8	8.0	6.7	6.4	34.6	>240.0	110,000	24,000	15.0	3,400	>240.0	0.7
10	1.3	27.0	8.0	0.1	6.3	12.4	4.6	>240,000	46,000	110.0	1,200	46.0	0.3

[a] Transect cruise July 1977. Station numbers correspond to those in Fig. 2.
[b] Dissolved oxygen.
[c] MPN/100 ml of water.
[d] Total viable count/1 ml of water.
[e] *V. cholerae* MPN/1 liter of water.

mation by a $\log_{10}$ transformation and analyzed for linear correlations, yielded no statistically significant associations for *V. cholerae* and the physical, chemical, and microbiological parameters examined (Table 3). *Salmonella* spp., however, exhibited a strong correlation, i.e., 1% level of significance, with fecal coliforms, total coliforms, total viable count, and suspended particulate matter.

Seasonal variation. *V. cholerae* distribution throughout the year was determined from samples collected at station 3, located at the mouth of the Chester River (Table 4). Seasonal variation was not observed, with the *V. cholerae* MPN remaining low and relatively constant throughout the year. During September 1977, samples were collected from two depths at station 3. A vertical salinity gradient was present at station 3 where brackish water of the Chester River layers over the higher-salinity Chesapeake Bay water. The top water sample collected 1 to 2 m below the surface demonstrated a salinity of 12.6‰, with a *V. cholerae* MPN of 0.4/liter. The bottom water sample, ca. 1 m above the sediment and approximately 11 m below the air-water interface, demonstrated a salinity of 16.6‰ and a *V. cholerae* MPN of 0.7/liter. In October, the salinity of the water was 4‰, with the range of water salinity for samples from which *V. cholerae* were isolated being extended to ca. 4–17‰.

Sediment and shellfish samples. Sediment samples were examined for the presence of *V. cholerae*. However, only one isolate, strain V45, was recovered from a sediment sample collected at station 9, located near Ft. McHenry in Baltimore Harbor. Oysters dredged at Eastern Bay (station 12) were examined for *V. cholerae*, and, of three separate sampling collections made during the summer of 1977, only one sample yielded *V. cholerae* (strain V49). Both the sediment and the oyster isolates were toxigenic when tested on the Y-1 cell culture.

Biochemical characterization. Results of selected biochemical characterization of the *V. cholerae* strains isolated from Chesapeake Bay are given in Table 5. All isolates gave typical reactions for oxidase (+), arginine dihydrolase (−), lysine decarboxylase (+), indole production (+), H_2S production (−), and gelatin hydrolysis (+). Variation among strains was observed for some carbohydrate fermentation reactions and the Voges-Proskauer test. Most of the isolates were identified as Heiberg group I or II with the remainder as Heiberg group V. No correlation between biochemical reactions and station location could be found.

Toxigenicity. Of 20 strains tested by the ligated RIL, 15 were positive. Fluid accumulation ranged from 0.21 to 2.11 ml/cm, with most of the results being less than 1 (Table 6). Good agreement between whole-culture response and cell-free culture filtrate was noted. In three instances, where whole-cell preparations did not cause fluid accumulation in the ligated RIL, cell-free filtrates were positive. Similarly, excellent correlation was noted between the RIL, mouse intraperitoneal inoculation, and Y-1 cell response. With few exceptions, the Y-1 cell response was uniformly positive. An additional 45 isolates also were examined, but only for Y-1 response, and these, in general, were also posi-

TABLE 3. *Correlation matrix of physical, chemical, and microbiological parameters*[a]

Parameter[b]	Trans	Temp	Salin	DO	pH	Sus mat	TVC	TV	TC	FC	Sal	Vp	Vc
Trans	1.0												
Temp	−0.76[c]	1.0											
Salin	0.64	−0.63	1.0										
DO	−0.33	0.66	−0.02	1.0									
pH	0.14	0.36	0.18	0.79[c]	1.0								
Sus mat	−0.66	0.14	−0.25	−0.05	−0.48	1.0							
TVC	−0.29	0.05	−0.57	−0.55	−0.63	0.51	1.0						
TV	−0.51	0.10	−0.50	−0.44	−0.72	0.75[c]	*0.92*[d]	1.0					
TC	−0.25	−0.24	−0.33	−0.65	−0.77[c]	0.71[c]	*0.88*	*0.91*	1.0				
FC	−0.16	−0.30	−0.16	−0.68	−0.77[c]	0.65	*0.86*	*0.90*	*0.96*	1.0			
Sal	−0.33	−0.21	−0.22	−0.45	−0.64	*0.86*	0.75[c]	*0.86*	*0.95*	*0.90*	1.0		
Vp	−0.34	−0.16	−0.25	−0.50	*−0.87*	0.62	0.60	0.80[c]	0.71[c]	0.71[c]	0.66	1.0	
Vc	−0.52	0.43	−0.09	0.17	−0.28	0.18	−0.10	0.17	−0.15	0.07	−0.16	0.45	1.0

[a] Transect cruise July 1977.

[b] Abbreviations: Trans, Transparency; Temp, temperature; Salin, salinity; DO, dissolved oxygen; Sus mat, total suspended matter; TVC, total viable count; TV, total *Vibrio* spp.; TC, total coliforms; FC, fecal coliforms; Sal, *Salmonella* spp.; Vp, *V. parahaemolyticus*; Vc, *V. cholerae*.

[c] Significant at the 0.05 level.

[d] Italics indicate significance at the 0.01 level.

tive, i.e., 39 positive and 6 either doubtful or negative (Table 6). Of the 65 isolates in the original test set, 57 (88%) were positive in the Y-1 adrenal cell cultures.

Serology. The Chesapeake Bay isolates of *V. cholerae* represent a variety of serotypes, as shown by the VRL (courtesy of Harry Smith). Of the 65 strains examined, 27 were not typable by any of the antisera currently available in the VRL collection. Selected serology data are given in Table 6.

DISCUSSION

The hypothesis that *V. cholerae* is an autochthonous bacterial species in brackish water has been presented by Colwell et al. (12). The data obtained in this study provide conclusive evidence that, indeed, *V. cholerae* can be isolated throughout the Chesapeake Bay in a distinct, not random, pattern dependent chiefly upon salinity and, apparently, inversely related to pollution. For example, at stations 1 and 2, in the upper Chesapeake Bay, where the salinity of the water was very low, viz., <4‰, *V. cholerae* were not isolated from samples collected at these stations. Although *V. cholerae* will grow in media prepared without added NaCl, growth is enhanced if media containing moderate amounts of salt, i.e., approximately 1% NaCl, are used. Conversely, *V. cholerae* will not grow in media prepared with a high concentration of salt.

Interestingly, the ecological data paralleled those of the laboratory studies, since no isolates were recovered from freshwater samples collected at the head of the Chesapeake Bay nor from the more saline regions of the lower Bay. There were no exceptions to the pattern. The moderately saline regions of the Bay consistently yielded *V. cholerae*. Thus, the optimal salinity limits for this organism in Chesapeake Bay are concluded to lie in the range of 4 to 17‰. It is interesting to note that the optimal salt concentration for *V. cholerae* in culture, i.e., approximately 1% (10‰), is that which approximates the in situ salinity range for this organism in middle portions of the Chesapeake Bay.

The distribution pattern for *V. cholerae*, as influenced by salinity, was further substantiated from results of analyses of samples collected in the James River, a subestuary of the Chesapeake Bay. The James River exhibits a salinity gradient similar to that of the Bay proper, i.e., freshwater at the head of the river and nearly seawater-strength salinity at the mouth. The pattern of distribution observed was that *V. cholerae* were not detected in regions of salinity extremes but were readily isolated where the water was of a moderate salinity. Other tributaries of the Chesapeake Bay are being studied to examine further the salinity-dependent distribution of *V. cholerae*.

TABLE 4. *Seasonal variation in V. cholerae MPN measured at station 3*

Date	*V. cholerae* MPN (cells/liter)	Temp (°C)	Salinity (‰)	TVC
May	1.1	17.2	6.4	2.6×10^3
July	0.7	27.5	9.9	1.6×10^4
September (TW)[a]	0.4	23.6	12.6	1.2×10^3
September (BW)[b]	0.7	25.2	16.6	1.0×10^3
October	1.5	12.7	4.0	4.4×10^3
November	<0.3[c]	11.3	6.1	1.1×10^4
December	0.4	4.4	6.3	8.2×10^3
January	0.9	0.2	4.9	4.9×10^4

[a] TW, Top water sample, ca. 1 to 2 m below air-water interface.

[b] BW, Bottom water samples, ca. 1 m above water-sediment interface.

[c] No *V. cholerae* were isolated.

TABLE 5. *Summary of biochemical reactions of V. cholerae strains isolated from Chesapeake Bay*

Test	Result (no. of strains out of 65 giving positive results)
Oxidase	65
Arginine dihydrolase	0
Lysine decarboxylase	65
Ornithine decarboxylase	64
Citrate utilization	63
H_2S production	0
Urease	0
Trytophan deaminase	0
Indole production	65
Gelatin hydrolysis	65
Growth in 0% NaCl	65
Voges-Proskauer	28
o-Nitrophenyl-β-D-galactopyranoside	65
Chitin digestion	58
Hemolysis	61
Acid from:	
Glucose	65
Mannitol	49
Inositol	0
Sorbitol	2
Rhamnose	0
Melibiose	0
Arabinose	0
Amygdalin	4
Sucrose +, Mannose + (Heiberg group I)	23
Sucrose +, Mannose − (Heiberg group II)	24
Sucrose −, Mannose + (Heiberg group V)	17

TABLE 6. *Toxicity data for selected Chesapeake Bay strains of V. cholerae*

Organism	Serotype	RIL			Mouse intraperitoneal inoculation (fatal within 18 h)	Y-1 adrenal cell response[a]
		No. of loops +/no. tested (whole culture)	Avg fluid accumulation in + loops (ml/cm)	No. of culture filtrate loops +/no. tested		
V-2	999[b]	2/3	0.45	1/3	2/2	+
V-3	999	1/2	0.56	1/2	2/2	+
V-4	24	2/2	0.35	1/2	2/2	+
V-5	23	2/2	0.63	1/3	0/2	+
V-10	14	2/2	0.73	0/2	2/2	+
V-11	999	0/3	—[c]	0/2	2/2	+
V-15	999	1/3	0.45		2/2	+
V-19	999	0/2	—	1/2	2/2	+
V-20	999	1/3	0.50	1/2	2/2	+
V-24	999	1/2	0.21	0/2	2/2	+
V-25	999	0/2	—	1/2	2/2	+
V-26	999	0/2	—	1/2	2/2	+
V-29	999	1/2	0.688	0/2	2/2	+
V-31	999	1/1	0.21	0/2	0/2	+
V-33	14	1/3	1.2	0/2	2/2	−
V-35	23	2/2	0.44	1/2	2/2	+
V-36	999	1/1	1.4	1/2	2/2	+
V-37	999	2/2	2.11	1/2	2/2	+
V-38	17	1/2	1.25	1/3	2/2	+

[a] An additional 45 isolates were also examined, of which 39 were positive and 6 were either doubtful or negative.

[b] 999 indicates strain not typable by any antiserum currently available in the VRL collection.

[c] No accumulation.

To obtain additional evidence of the autochthonous nature of *V. cholerae* in the Chesapeake Bay, a pollution gradient of water samples was examined for presence of *V. cholerae*, with the objective being to determine association, if any, with recommended microbial indicators of pollution. *Salmonella*, a pathogen found in natural waters where sewage discharges occur, was isolated only from samples collected in the highly polluted Baltimore Harbor, where significant numbers of fecal coliforms were also found. In one instance, the *Salmonella* MPN was observed to be greater than 240/100 ml, the highest reported value for samples collected in an estuarine environment. Salmonellae have been also isolated from samples collected in the harbor of Little Creek, Va., where the salinity is relatively high, ca. 21‰ (R. R. Colwell and J. Kaper, Proceedings of the Fourth Biennial International Estuarine Research Conference, in press). However, fecal coliform counts in the water at Little Creek were also high, i.e., approximately that of the Baltimore Harbor stations from which isolation of *Salmonella* spp. was reported (24; R. R. Colwell and J. Kaper, in press). Results of a correlation matrix, which are summarized and are given in Table 3, indicate that the presence of *Salmonella* is indeed associated with elevated numbers of fecal coliforms ($P < 0.01$).

In contrast to the *Salmonella* spp., which can be considered allochthonous pathogens, *V. cholerae*, as does *V. parahaemolyticus* (10), appears to be an autochthonous species in the estuarine ecosystem. *V. cholerae* were found to be widely distributed throughout the Chesapeake Bay, within a certain salinity range, both in polluted and in unpolluted areas, and the organism's incidence was not found to be correlated with other microbial indicators of pollution. Indeed, the converse was indicated. For example, *V. cholerae* organisms were not isolated during the summer of 1977 at the heavily polluted site, Jones Falls in Baltimore Harbor. The initial isolation of *V. cholerae* was made at Jones Falls in Baltimore Harbor during October 1976 (11). Since that time, mixed success has been achieved in isolating *V. cholerae* from samples collected at Jones Falls.

Jones Falls, a location where the salinity of the water lies within the range of 4 to 17‰, often demonstrates nearly anoxic water conditions, as indicated by dissolved oxygen content, viz., 0.1 mg/liter (Table 2). Large numbers of allochthonous bacterial species, viz., 240,000 total coliforms/100 ml, 46,000 fecal coliforms/100 ml, and total viable, heterotrophic counts of 1.1×10^7/ml were obtained.

Although *V. cholerae* strains were recovered

from sediment and oyster samples, these were sporadic in incidence, and quantification could not be made. Because of the low numbers of *V. cholerae* present in the water and the resulting need for sample concentration, it is concluded that *V. cholerae* are present in sediment and shellfish in low numbers, with consistent recovery not possible unless large volumes of such samples can be processed efficiently.

Seasonal monitoring was accomplished at the mouth of the Chester River, at station 3, where *V. cholerae* were recovered at temperatures as low as 0.2°C. The number of *V. cholerae* in the samples did not change significantly throughout the year. Thus, a seasonal effect could not be demonstrated for *V. cholerae,* in contrast to *V. parahaemolyticus,* for which a seasonal cycle in Chesapeake Bay has been established (13, 23a). Interestingly, *V. parahaemolyticus* could not be isolated from water samples collected in winter months, but the species was found to overwinter in the sediment. When temperatures rise in late spring, the vibrios are released from the bottom communities to enter the water column, most probably via attachment to zooplankton. A similar cycle may exist for *V. cholerae,* but preliminary results are not strongly supportive of that hypothesis, instead indicating that the organism can be detected in the water column at the same low levels during the period of colder temperatures as well as during the summer months. *V. cholerae* strains isolated from Chesapeake Bay are capable of digesting chitin, lending credibility to an association with zooplankton since chitin is a major structural component of many aquatic invertebrates. Studies designed to elucidate the association, if any, of *V. cholerae* with zooplankton have not yet been done, but are planned.

In this study, biochemical characteristics of strains identified as *V. cholerae* were found to be in close agreement with those reported by Sakazaki et al. for non-group O-1 *V. cholerae* (36), and these strains can be classified according to fermentation pattern (20), although such groupings apparently are not significant in terms of pathogenicity, ecology, or serology. In general, *V. cholerae* are considered to be arabinose negative (44), further diminishing the usefulness of the Heiberg scheme. Most of the isolates from Chesapeake Bay were found to belong to Heiberg groups I or II, with several strains in group V.

Toxicity data for the Chesapeake Bay strains of *V. cholerae* were found to be comparable to results of studies reported by Ohashi et al. (31) and Zinnaka and Carpenter (48), who examined strains of *V. cholerae* isolated from clinical specimens collected in Southeast Asia and Sudan, namely that fluid accumulation was substantially less than that induced by classical *V. cholerae* O-1 strains. Interestingly, the Chesapeake Bay vibrios appeared to be more strongly positive in the pathogenicity tests when the cultures were grown statically, an interesting observation since Ohashi et al. (3) and Zinnaka and Carpenter (48) examined cultures for pathogenicity that had been grown in broth culture with shaking.

The serotypes of *V. cholerae* isolates from Chesapeake Bay are of wide geographic occurrence. The 38 typable strains represented serotypes previously isolated from a variety of specimens throughout the world. For example, strains V33, V52, and V54, isolated from stations 9, 14, and 19, respectively, were serotype 14. Other strains of serotype 14 have been recovered from night soil in Bangladesh, sewage in Hungary, and a case of diarrhea in the Philippines (H. Smith, personal communication). Strain V38 was isolated from the Chester River, station 3, and was classified as serotype 17. Other strains of serotype 17 have been recovered from nonenteric infections in Czechoslovakia, night soil in Hong Kong, sewage in Iraq, and a case of diarrhea in the United States. It should be noted that there was no discernible correlation of serotype and pathogenicity or station location.

Clearly, the significance of the findings reported here is that an organism not considered to be epidemiologically significant in the United States has been found to be indigenous to Chesapeake Bay, a major natural water system. Most of the strains isolated from the Chesapeake Bay demonstrated positive results in the toxigenicity tests that were done. The numbers of enterotoxin-producing *V. cholerae* in Chesapeake Bay, however, were very low. Cash and co-workers demonstrated that approximately 10^8 cells of classical *V. cholerae* are required before clinical evidence of cholera is manifested in volunteers possessing normal gastric acidity (7). A higher stomach pH was conducive to infection at lower concentrations, i.e., ca. 10^4 cells. The numbers of *V. cholerae* found in Chesapeake Bay water were in the range 1 to 10/liter, significantly less than that required for infection if direct ingestion were to occur.

Shellfish are more likely to be a source of infection than water, since shellfish are filter-feeding organisms, thereby capable of concentrating bacteria. Indeed, in the case of cholera that occurred in Alabama in 1977, the exact source of infection was unknown, but it was noted that the patient had a history of eating large quantities of raw oysters (5). Localized concentrations of *V. cholerae* conceivably can

develop in shellfish in the Chesapeake Bay, with the consequence of inducing diarrheal disease in susceptible individuals. The strain of *V. cholerae* isolated from Chesapeake Bay oysters was toxigenic by methods employed in this study. Reports of shellfish-borne cholera outbreaks caused by both *V. cholerae* group O-1 and *V. cholerae* of serotypes other than O-1 have been published (4, 15).

Several strains of *V. cholerae* of serotype other than O-1 have been isolated in the United States. Since 1972, there has been a dramatic increase in the number of reports involving infections associated with *V. cholerae* serotypes other than O-1 (23). Of 26 isolates reported by the Center for Disease Control, 50% were isolated from feces of patients with gastroenteritis, 9 (35%) were isolated from other body fluids and tissues, such as sputum, blood, and gall bladder, and 4 deaths resulted from these infections (23). It was found that patients with gastroenteritis caused by *V. cholerae* strains other than O-1 frequently had a history of recent shellfish ingestion and/or foreign travel. Some of the patients with systemic *V. cholerae* infection had occupational or recreational exposure to salt water, a pattern of the disease underscoring the significance of the widespread distribution of *V. cholerae* in the Chesapeake Bay. Indeed, one of the cases reported by Center for Disease Control involved a patient who lived on the shore of the Chesapeake Bay. Interestingly, 73% of the 26 patients infected with these strains live in coastal states, thus suggesting a wide geographical significance to the discovery of *V. cholerae* in Chesapeake Bay, the implication being that this organism is a resident of brackish waters, regardless of geographical location.

The case of cholera in Texas in 1973 occurred in Port Lavaca, a city on the Texas Gulf Coast, and an extensive environmental investigation was carried out afterwards (46). Although *V. cholerae* O-1 was isolated from the stool of the patient, no *V. cholerae* of serotype O-1 could be isolated from contacts or environmental sources, and no source of infection was determined. Several strains of toxigenic *V. cholerae* of serotype other than O-1 were recovered from the environment, and the possibility that these were capable of "mutating" to O-1 or vice versa has been considered (46). It has previously been reported that at the end of some cholera epidemics, strains of *V. cholerae* O-1 have been supplanted by *V. cholerae* strains of serotypes other than O-1 (19). Conversely, the cholera outbreak in Hong Kong in 1964 was preceded by an increased incidence of *V. cholerae* of serotypes other than O-1 (H. W. Wyile, M.D. thesis, University of Manchester, Manchester, England, 1968). Whether or not "transformation" of somatic antigen can occur in the natural environment is not known. Nevertheless, the critical point is that *V. cholerae* of serotypes other than O-1 can be pathogenic and should be regarded as potential pathogens.

The data accumulated to date for *V. cholerae* isolated from Chesapeake Bay support the currently accepted taxonomy of *V. cholerae*, whereby both agglutinable and non-agglutinable, as well as pathogenic and non-pathogenic serotype O-1 strains are included in the genospecies and taxospecies (38, 44). *V. parahaemolyticus* has been excluded from the genus *Vibrio* on the basis of ionic requirement and habitat by Baumann and Baumann (2). Results of the present study establish that the natural habitat of *V. cholerae* appears to be natural bodies of water. *V. parahaemolyticus* has been renamed *Beneckea parahaemolyticus* by Baumann and Baumann because of their claim that its natural habitat was the aquatic environment, whereas *V. cholerae* was concluded by them to be restricted to human association (2). Clearly, from the results of this study, the assumptions of Baumann and Baumann regarding the ecology of *V. cholerae* should be reevaluated. In addition, from our work on the systematics of *V. cholerae* and *V. parahaemolyticus*, based on numerical taxonomy, DNA base composition, and DNA/DNA reassociation data (8, 9), we concluded that *V. cholerae* should not be separated from *V. parahaemolyticus* at the genus level, but, rather, that both species be retained within the genus *Vibrio*. Interestingly, Baumann and Baumann (2) observed that *V. cholerae* is more closely related, by ribosomal RNA homology, to the "marine enterobacteria" than to terrestrial enterobacteria, a finding readily understood if *V. cholerae* is indeed considered to be a natural inhabitant of the brackish water areas of estuaries.

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ADDENDUM

The salinity-dependent distribution of *V. cholerae*

in Chesapeake Bay described in the study reported here has been confirmed by more recent work carried out in the Potomac River, a major tributary of the Bay. Also, in additional studies in Chesapeake Bay, carried out after completion of this manuscript, two isolates of *V. cholerae* were recovered from water samples the salinity of which measured 1 and 3‰, respectively. Thus, the lower level of salinity governing distribution of *V. cholerae* in brackish waters may not be so stringent, with isolation of the organism being possible in natural environments of salinities <4‰ but with the optimum salinity lying, in general, in the range of 4 to 17‰.

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