Journal of Food Protection, Vol. 46, No. 9, Pages 802-806 (September 1983)
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Non-O1 *Vibrio cholerae* in Shellfish, Sediment and Waters of the U.S. Gulf Coast

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(Received for publication January 3, 1983)

ABSTRACT

In a study conducted throughout U.S. Gulf Coastal waters, *Vibrio cholerae* non-O1 was isolated more frequently from water samples than from shellfish or sediment samples. Frequency of *V. cholerae* recovery was directly related to water temperature and inversely related to salinity. The presence of *V. cholerae* was not adequately indicated by the fecal coliform standards for shellfish-growing waters and market shellfish as established by the National Shellfish Sanitation Program. Although all cultures tested by the Y-1 mouse adrenal cell assay or by radioimmunoassay for production of a cholera toxin-like toxin were negative, 4 of 13 isolates caused diarrhea in the infant rabbit.

During a cholera outbreak in Louisiana in August and September 1978, *Vibrio cholerae*, El Tor, Inaba, was isolated from the stools of 11 infected individuals who had consumed domestically prepared boiled or steamed crabs; the organism was also isolated from some leftover crab (*4,5*). This cholera outbreak, the first of any significance in the United States since 1911, was caused by a *V. cholerae* O1 serotype, i.e., one which agglutinates with O group 1 antisera. Gastroenteritis and other infections caused by non-O1 serotypes, i.e, those that do not agglutinate in O group 1 antisera, have received greater recognition in recent years (*3,7,8,10,14,19*). A review of recent cases of non-O1 *V. cholerae* gastroenteritis acquired in the U.S. reports that all were associated with consumption of raw oysters (*18*). The distribution of *V. cholerae* non-O1, which has been isolated throughout the Chesapeake Bay area, is thought to be influenced by salinity (*16*).

This study examined the prevalence of various strains of *V. cholerae* in shellfish, sediments and waters along the U.S. Gulf Coast; the relationship between *V. cholerae* and salinity or temperature of environmental waters; the adequacy of fecal coliform levels as indicators of *V. cholerae* in environmental samples; and the toxigenicity and pathogenicity of *V. cholerae* isolates.

MATERIALS AND METHODS

Sampling sites

Between October 1978 and June 1980, 21 stations from Texas to Florida were sampled. Shellfish and water samples were collected from Galveston Bay, TX (2 sites); Mississippi Sound, MS (4 sites); Mobile Bay, AL (3 sites); Mississippi River Delta, LA (6 sites); Panama City, FL (1 site); Apalachicola Bay, FL (3 sites); and Tampa Bay, FL (2 sites). Sediment samples were collected from sites in Louisiana and Mississippi. In general, samples were collected monthly; however, special samples from areas with reported cholera cases were also analyzed.

Collection and handling of samples

Water samples were collected in sterile 4-L wide-mouth Nalgene containers by the method of the American Public Health Association (*1*). Shellfish samples were collected with oyster tongs or by dredging. Sediment samples, also collected with oyster tongs, were aseptically placed in sterile 250-ml polypropylene beakers with lids. Samples were iced and stored 24 h before analysis. Ice was contained in plastic bags or ice packs to prevent direct contact of shellfish with ice or ice water. Surface water temperature (mercury thermometer) and salinity (hand refractometer, American Optical Corp.) were determined for each sampling site.

Bacteriological analyses

Fecal coliform levels in seawater were estimated by the A-1 method (*2*), which uses a 5-tube most probable number (MPN) technique. Shellfish and sediment fecal coliform levels were determined with lauryl sulfate tryptose (LST) and EC broths (Difco) by the 5-tube MPN technique (*1*).

Vibrio cholerae *analysis*

Shellfish and sediment samples for *V. cholerae* were analyzed by a modification of the method outlined in the *Bacteriological Analytical Manual* (BAM) (*11*). Shellfish were scrubbed, shucked and blended for 1 min in a Waring Blendor at 14,000 rpm. Two 100-g portions of the homogenate were mixed by swirling in separate 2-L Erlenmeyer flasks, each containing 900 ml of alkaline peptone water (10 g Bactopeptone/L (Difco) and 10 g NaCl/L, pH 8.5). Two 100-g portions of sediment were similarly mixed. Before incubation, a loopful of the alkaline peptone water suspension was streaked onto each of three plates of thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco) and gelatin agar (22). The al-

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TABLE 1. Vibrio cholerae *isolation in water, shellfish and sediment samples at selected U.S. Gulf Coast estuaries.*

Location	Water samples		Shellfish samples		Sediment samples	
	No. of stations sampled[a]	No. of samples collected	No. of stations sampled	No. of samples collected	No. of stations sampled	No. of samples collected
Galveston Bay, TX	2(100.0)	17(76.5)	2(100.0)	30(20.0)	0 -	0 -
Mississippi River Delta, LA	5(100.0)	67(73.1)	6(100.0)	78(38.5)	5(100.0)	62(27.4)
Mississippi Sound, MS	4(100.0)	36(79.3)	3(66.7)	36(19.4)	3(66.7)	28(7.9)
Mobile Bay, AL	2(100.0)	24(54.2)	3(66.7)	27(22.2)	0 -	0 -
Panama City, FL	1(100.0)	7(57.1)	1(100.0)	12(8.3)	0 -	0 -
Apalachicola Bay, FL	3(100.0)	15(86.7)	3(0.0)	15(0.0)	0 -	0 -
Tampa Bay, FL	2(50.0)	8(25.0)	2(0.0)	15(0.0)	0 -	0 -
Totals	19(94.7)	174(71.3)	20(65.0)	213(24.9)	8(87.5)	90(21.1)

[a]Numbers in parentheses are % positive for *V. cholerae*.

kaline peptone water suspension was incubated for 6 to 8 h at 35°C for enrichment of *V. cholerae*, and streaking of material from the pellicle was repeated on additional TCBS and gelatin agar plates. Both sets of plating media were incubated at 35°C for 18 to 24 h. Bacterial colonies of diverse morphology were transferred from each medium and streaked for purification on T_1N_1 agar plating medium containing 10 g tryptone/L (Difco), 10 g NaCl/L and 20 g agar/L (Difco). Suspect cultures were screened and confirmed biochemically by the method outlined in the BAM, except that the modified decarboxylase dihydrolase medium of Smith and Bhat-Fernandes (*23*) was used for amino acid reactions. This medium gave more distinct reactions than the medium recommended by BAM.

Water samples were analyzed for *V. cholerae* by the membrane filter-MPN procedure (*20*). The microflora from 2-L portions of seawater were concentrated by vacuum filtration on a 142-mm diameter Millipore filter (0.45μm). Celite (Johns-Mansville), a filtering aid, was suspended in distilled water (1 g/100 ml), autoclaved at 121°C at 15 psi for 15 min and added to the samples before filtration. Filters were blended at high speed for 1 min in 100 ml of alkaline peptone water to create a 20-fold concentration of the microflora. A series of decimal dilutions (10 ml through 0.001 ml) was prepared from this suspension, and appropriate amounts were inoculated into tubes containing 10-ml portions of alkaline peptone water for 5-tube MPN analysis. After 18 to 24 h of incubation at 35°C, a loopful of material from the pellicle of each alkaline peptone tube showing growth was streaked onto one plate each of TCBS agar and gelatin agar. From this point, the analysis was the same as that used for shellfish and sediments.

Toxicity studies

Isolates were maintained on T_1N_1 slants covered with sterile mineral oil. Enterotoxigenicity of suspect *V. cholerae* isolates was determined by the Y-1 mouse adrenal cell method (*21*) and by the radioimmunoassay (RIA) method of Greenberg et al. (*12*).

Pathogenicity study

Pathogenicity was determined with the infant rabbit model by the following method. Test and control *V. cholerae* cultures were inoculated into casamino acid-yeast extract broth (Difco) and incubated at 37°C on a roller tube assembly for 23 to 24 h. The broth cultures were transferred to sterile centrifuge tubes and spun under refrigeration at 24,000 × *g* for 20 min. The supernatant fluid was discarded and the cells were resuspended in phosphate-buffered saline, pH 7.0, to give a concentration of approx. 10^9 organisms/ml.

Within 6 to 7 h after being taken from the mother, 8-to 10-d-old rabbits were anesthetized; an incision was made in the abdominal cavity of each animal and 1 ml of cell suspension was injected into the ileal region of the small intestine. The incisions were sutured and the animals were returned to their cages. The rabbits were sacrificed after 24 ± 1 h and the cecum and intestines were examined for gross fluid accumulation. Heavy diarrhea in the cage was additional evidence that the animal had experienced a cholera-like infection. A control animal which had been inoculated with a known human pathogen 569B (stool, India; obtained from Robert M. Twedt, Food and Drug Administration, Cincinnati, OH) was included with each group of rabbits tested.

Serotyping of isolates

Live organisms were used to type suspect cultures; the O1 antiserum was provided by Dr. Harry Smith of the Vibrio Reference Laboratory, Thomas Jefferson Medical College, Philadelphia, PA.

Statistica analysis

Data grouped within temperature, salinity and fecal coliform ranges were reduced by analysis of variance (*24*), by the mean separation technique of Duncan (*9*) and by Fisher's exact one-tailed test (*17*).

RESULTS

Prevalence

The prevalence of *V. cholerae* in a total of 477 water, shellfish and sediment samples from various Gulf Coast locations is given in Table 1. Biochemical profiles identified 963 isolates as *V. cholerae*. Only two isolates (one from oysters and one from water) were agglutinable with O1 antiserum. *V. cholerae* was isolated from 71.3% of 174 seawater samples, 24.9% of 213 shellfish samples and 21.1% of 90 sediment samples. The organism was found in all the estuarine systems and was isolated from seawater, shellfish or sediment samples of 19 of the 21 stations. The two stations from which *V. cholerae* was not recovered were sampled only once.

The frequency of isolation of *V. cholerae* in seawater samples was 50% for all locations except Tampa Bay, where one of two stations was sampled only once and no *V. cholerae* was isolated. Frequency of *V. cholerae* recovery from shellfish was greatest at stations in the Mississippi River Delta of Louisiana. *V. cholerae* was not isolated from shellfish in Tampa or Apalachicola Bay, and was isolated only once from the Panama City area.

Some stations were not sampled systematically to cover wide ranges of salinity and temperature. These variables may have influenced the general incidence and frequency of *V. cholerae* recovery in certain locations and are discussed below.

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TABLE 2. *Effect of water temperature on isolation of* V. cholerae *from water, shellfish and sediment samples.*

Temperature range (°C)	No. of samples collected	% Positive for *V. cholerae*	*V. cholerae* isolates/ sample	*V. cholerae* MPN/L (log_{10})
		Water samples		
≤15	40	52.5[a]	2.28[c]	1.20[c]
16-20	36	77.8[b]	3.75[c,d]	1.44[c]
21-25	29	79.3[b]	4.62[c,d]	2.37[d]
≥26	69	75.4[b]	5.26[d]	2.81[d]
		Shellfish samples		
≤15	65	10.8[a]	0.31[c]	
16-20	48	12.5[a]	0.27[c]	
21-25	36	25.0[a]	0.75	
≥26	64	51.6[b]	2.05[d]	
		Sediment samples		
≤15	26	7.7[a]	0.08[c]	
16-20	21	0.0[a]	0.00[c]	
21-25	10	20.0[a,b]	0.60[c,d]	
≥26	33	45.5[b]	1.24[c]	

[a,b]Mean values in the same column followed by a common letter are not significantly different ($P<0.05$) by Fisher's exact one-tailed test.

[c,d]Mean values in the same column followed by a common letter are not significantly different ($P<0.05$) by Duncan's mean separation technique.

TABLE 3. *Effect of water salinity on isolation of* V. cholerae *from water, shellfish and sediments.*

Salinity range (ppt)	No. of samples collected	% Positive *V. cholerae*	*V. cholerae* isolates/ sample	*V. cholerae* MPN/L (log_{10})
		Water samples		
≤5	52	80.8[a]	6.65[c]	2.28[c]
6-10	56	75.0[a]	4.34[d]	2.33[c]
11-15	45	71.1[a]	2.40[d,e]	1.99[c,d]
≥16	21	38.1[b]	1.24[e]	1.17[d]
		Shellfish samples		
≤5	68	32.3[a]	1.01[c,d]	
6-10	57	38.6[a]	1.86[c]	
11-15	54	14.8[b]	0.22[d]	
≥16	34	8.8[b]	0.12[d]	
		Sediment samples		
≤5	24	25.0[a]	0.88[c]	
6-10	36	25.0[a]	0.61[c]	
11-15	26	15.4[a]	0.23[c]	
≥16	4	0.0[a]	0.00[c]	

[a,b]Mean values in the same column followed by a common letter are not significantly different ($P<0.05$) by Fisher's exact one-tailed test.

[c,d,e] Mean values in the same column followed by a common letter are not significantly different ($P<0.05$) by Duncan's mean separation technique.

Influence of water temperature on prevalence of V. cholerae

V. cholerae levels in water, shellfish and sediment samples within specified water temperature ranges are shown in Table 2. The frequency of isolation of *V. cholerae* from water samples was substantially increased when the temperature was greater than 15°C; significantly higher concentrations of *V. cholerae* (MPN/L, $P\leq0.05$) were observed when the water temperature exceeded 20°C. Shellfish and sediment samples followed a similar pattern. When the water temperature exceeded 25°C, isolation was more than twice as likely than it was in shellfish and sediment from cooler waters.

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TABLE 4. Vibrio cholerae *isolation in NSSP approved and prohibited shellfish-growing waters and market shellfish.*

Fecal coliform MPN range[a]	No. of samples collected	% Positive *V. cholerae*	*V. cholerae* isolates/ sample	*V. cholerae* MPN/L (log_{10})
		Water samples		
≤ 14	76	67.1	3.92[b,d]	1.87[b,d]
> 14	98	74.5	4.34[b,d]	2.25[b,d]
		Shellfish samples		
≤230	94	19.1	0.45[c,d]	-
>230	119	31.1	1.25[c,e]	-

[a]MPN values based on 100 ml for water samples; 100 g for shellfish samples.
[b]Fecal coliform MPNs of water samples based on results in A-1 medium at 44.5°C for 24 h.
[c]Fecal coliform MPNs of shellfish samples based on results of APHA method using LST and EC broths.
[d,e]Mean values in the same column followed by a common letter are not significantly different ($P<0.05$) by Duncan's mean separation technique.

Influence of salinity on prevalence of V. cholerae

V. cholerae levels in water, shellfish and sediment samples within specified salinity ranges are shown in Table 3. The percentage of water samples in which *V. cholerae* was isolated dropped when salinity exceeded 15 ppt. *V. cholerae* concentrations in water samples measured by MPN/L or isolates recovered per sample generally increased with decreasing salinity, with significant differences ($P=0.05$) between samples in the highest and lowest salinity categories. A similar inverse relationship between salinity and *V. cholerae* levels occurred for shellfish and sediment samples.

Relationship between fecal coliform and numbers of V. cholerae

The National Shellfish Sanitation Program (NSSP) uses the fecal coliform standard to classify shellfish-growing waters. If the median fecal coliform MPN (5 tubes, 3 dilutions) of an area exceeds 14/100 ml, or if more than 10% of the samples exceed an MPN of 43/100 ml, shellfish harvesting is prohibited in that area (*15*). There is no NSSP fecal coliform standard for shellfish meats in growing areas; however, the market standard for shellfish meats is an MPN of 230 fecal coliforms/100 g of meat. Table 4 lists mean *V. cholerae* MPNs in seawater and shellfish relative to NSSP fecal coliform limits. *V. cholerae* levels were not significantly different ($P\leq0.05$) between seawater samples with MPNs ≥ and ≤14. Significantly fewer ($P\leq0.05$) *V. cholerae* isolates were recovered from shellfish meats with a fecal coliform MPN ≤230; however, 19.1% of shellfish meat samples acceptable by the NSSP market standard were positive for *V. cholerae*.

Toxicity and pathogenicity of V. cholerae *isolates*

A total of 781 cultures were tested for production of a cholera toxin-like (CT-like) toxin by the Y-1 mouse adrenal cell assay and 656 by radioimmunoassay (RIA). Although none was toxigenic, 3.3% of those tested by the Y-1 mouse adrenal cell assay caused a cytopathogenic effect on the cell culture. Of 13 isolates tested in the infant rabbit model, 4 caused diarrhea.

DISCUSSION

The widespread isolation of *V. cholerae* non-O1 and the sporadic recovery of *V. cholerae* O1, Inaba, in this study was not unexpected. Other researchers (*6,13,25*) have reported the isolation of *V. cholerae* at various Gulf Coast locations but have not provided data on relative levels between major estuaries on the Gulf Coast. Relatively low levels of *V. cholerae* in samples from Tampa Bay and from areas near Panama City were probably due to high salinity. Failure to isolate *V. cholerae* from Apalachicola Bay shellfish was probably due to a combination of sampling over a limited time and the inadequacy of the BAM methodology.

An extensive study of the Apalachicola Bay area conducted over a wide range of environmental conditions has shown that *V. cholerae* is present in shellfish samples at levels similar to those of other Gulf Coast estuaries (DePaola et al., manuscript in preparation). The discrepancy between isolation frequency of *V. cholerae* in shellfish and water samples is most obvious in Apalachicola Bay samples but is evident in all locations.

Although temperature-dependent distribution of *V. cholerae* was not observed in a 1979 study of Chesapeake Bay (*16*), significant increases in *V. cholerae* levels in seawater, shellfish and sediments with increasing water temperatures were demonstrated in the present study (Table 2). In addition, an inverse relationship between salinity and *V. cholerae* levels was found to exist in all sample types. *V. cholerae* was recovered from seawater samples with salinities ranging from 0 to 30 ppt, with the highest levels found at salinities ≤5 ppt. This contrasts with the Chesapeake Bay study (*16*) in which *V. cholerae* was isolated only at salinities of 4 to 17 ppt.

The level of fecal coliforms in seawater samples was not indicative of the level of *V. cholerae*. There was a direct

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and significant relationship between the NSSP market standard and *V. cholerae* levels in shellfish meats; however, some shellfish which met the NSSP market standards were found to contain *V. cholerae*.

The prevalence of *V. cholerae* in the marine environment has been well-documented with the public health significance of these organisms as the primary concern. A CT-like toxin, the major cause of cholera diarrhea, can be detected by the Y-1 mouse adrenal cell assay. None of the Gulf Coast environmental isolates tested by the Y-1 mouse adrenal cell assay or by RIA produced CT-like toxin. Although only a limited sample of isolates was tested in the infant rabbit model, the occurrence of diarrhea in four rabbits suggests that some environmental isolates can cause disease.

Further testing of Gulf Coast isolates which cause a cytopathogenic effect in Y-1 mouse adrenal cells should be tested in various animal models to provide possible clues about their pathogenic potential.

ACKNOWLEDGMENTS

For collection of the environmental samples, we thank the Texas State Department of Health, Division of Marine Resources; the Louisiana Department of Health and Human Resources, Office of Health Services and Environment Quality, Water Quality Section; the Mississippi State Board of Health, Bureau of Environmental Health, Milk and Shellfish Division; and the Florida Division of Natural Resources, Shellfish Sanitation Branch. We also thank M. Dixon Boswell, Charles Goodwin and Mabel Carter for collection and analysis of the bacterial samples; Foster McClure, Division of Mathematics, Food and Drug Administration, Washington, DC, for statistical analysis of the data; Dr. Robert M. Twedt, Food and Drug Administration, Cincinnati, Ohio, for providing the human pathogen 569B; and Dr. Harry Smith, Vibrio Reference Laboratory, Thomas Jefferson Medical College, Philadelphia, PA, for providing 01 antisera and for serotyping the suspected *V. cholerae* non-01 isolates.

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