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# Enumeration of *Vibrio* Species, Including *V. cholerae*, from Samples of an Oyster Growing Area, Grays Harbor, Washington

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ABSTRACT

Water, shellfish, and sediment samples from Grays Harbor, a major commercial oyster producing estuary in the State of Washington, were examined for levels of *Vibrio* species. Non-01 *V. cholerae* was found at low levels in 37.8% of the samples. While *V. parahaemolyticus* was found in all samples, levels were low. *V. mimicus* and *V. fluvialis* were found infrequently and at low levels. Potentially pathogenic strains of non-01 *V. cholerae* and Kanagawa positive *V. parahaemolyticus* were isolated from oysters suggesting a potential for human illness.

In the past decade the incidence of *V. cholerae* has been extensively studied in estuaries of the U.S. (*2,5,7,9,11,13,15*). It has been concluded from these studies that non-01 *V. cholerae* is an autochthonous component of the estuarine microbial community. Waters off the West Coast of U.S. have recently been studied and a high incidence of *V. cholerae* has been reported (*7,9,15*). However, only limited studies of the incidence of *V. cholerae* in West Coast shellfish have occurred with only one report from multiple species (*7*).

Grays Harbor is a significant commercial production area for oysters, *Crassostrea gigas*, in the State of Washington. Results of a recent study (*7*) indicate that non-01 *V. cholerae* was present in half of all oyster samples analyzed from the West Coast. The purpose of this study was to enumerate *Vibrio* species from water, oyster, and sediment samples collected during a National Shellfish Sanitation Program (NSSP) certification study of the estuary.

## MATERIALS AND METHODS

### *Sampling*

Forty-five samples were collected in September over a 10 d period from 16 monitoring stations of the State of Washington Department of Social and Health Services, Shellfish Program (Fig. 1). Surface water was collected in sterile 4L plastic bottles (Nalge Co., Rochester, N.Y.) from growing areas and tributaries. Oyster shellstock was collected by dredge or tong from the beds and placed in sterile plastic bags. Sediment was collected using a sterile 5 cm diameter plastic core. Approximately 10 cm of core was placed in sterile "Whirl-Pak" (Nasco West, Modesto, CA) bags. Samples were placed on ice, transported to a mobile laboratory and analyzed within 6 h. Water temperature and salinity were measured at the time of collection by salinometer.

### *Bacteriological*

Fecal coliforms were enumerated by the most probable number technique (MPN) with confirmation by standard methods (*1*). Oyster shellstock was scrubbed, shucked, and blended undiluted by the standard procedure. Sediment was mixed within the bag and diluted, as were oyster homogenates, with sterile 2% NaCl, pH 7, diluent.

*Vibrio* species were enumerated by an MPN technique with 18 h enrichment at 35°C in Alkaline Peptone Water (APW) (*3*). Three liters of each water sample were filtered through 0.45 micron, 142 mm diameter sterile membranes (Millipore Corp., Bedford, MS). Membranes were placed in 100 ml volumes of APW, blended 1 min, and the homogenate dispensed to tubes of APW for 3-tube, 3 dilution MPN. Dilutions of sediments and oyster were dispensed directly to APW tubes.

After 18 h incubation, contents of APW were streaked to Thiosulfate Citrate Bile Salts (TCBS, Oxoid USA) and to Polymyxin-Mannose Tellurite (PMT, Nissiui, Tokyo, Japan) selective agars. Suspect colonies were picked from agars after 18-24 h incubation at 35°C. Isolates were screened with a modified arginine based medium (*6*) and identification procedures were as described (*3*).

*V. cholerae* were serotyped using polyvalent 01 Inaba and Ogawa sera supplied by Dr. Harry Smith, Vibrio Reference Laboratory, Philadelphia, PA. Seventeen isolates, 12 *V. cholerae* and 5 *V. mimicus*, were tested for cholera toxin (CT) and cytotoxic activity using the Y-1 mouse adrenal cell (CCL79, ATCC, Rockville, MD) assay (*3*).

Five hundred sixty-six sucrose negative isolates were tested for the thermostable direct hemolysin (TDH) of *V. parahaemolyticus* and cytotoxin hemolysin gene of *V. vulnificus* by DNA colony hybridization procedures by gene probe. Colony blots were made from overnight, 35°C growth of isolates on Tryptic Soy Agar-2% NaCl plates using Whatman 541 filters (*10*). Colony lysing and DNA hybridization procedures followed those previously described (*3*). Radiolabeled probe for the TDH-3 sequence of *V. parahaemolyticus* (*12*) and the cytotoxin-hemolysin of *V.*

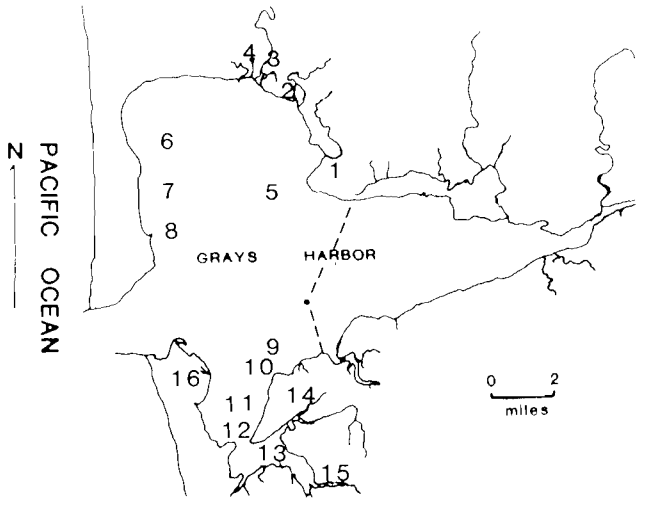


Figure 1. *Sampling stations of commercial oyster beds in Grays Harbor, Washington. Dotted line indicates the prohibited harvesting area in the eastern portion of the estuary.*

*vulnificus* (*16*) was labeled with 32P and supplied by W. E. Hill, CFSAN, FDA, Washington, D.C. Control strains were *V. parahaemolyticus* NY 477 (Kanagawa positive) and *V. vulnificus* LA M624, both clinical isolates.

## RESULTS AND DISCUSSION

Non 01 *V. cholerae* was isolated from 17 (14 water, 3 oyster) of the 45 samples (37.8%), while *V. mimicus* was limited to 5 samples, including 1 oyster (Table 1). Water samples were more productive for *Vibrios* than either oyster or sediment samples, confirming data reported in another environmental study (*2*). Most of the *V. cholerae* isolations, 15 samples, were from the north part of the estuary, Stations 1-8, as well as the 5 samples containing *V. mimicus*. Station 5 was the most productive followed by Stations 1-4, which may be due to the hydrography of the bay, namely the drainage pattern and tidal flushing of this shallower portion of the estuary.

Generally, low levels of *V. cholerae*, and particularly *V. mimicus*, were found. Counts ranged from 0.1 to 40 MPN/100 ml of sample, with 0.1 to 0.3/100 g found in oysters. Similarly, low levels of *V. cholerae* in waters (*9*), and sediments (*15*) have been reported for other estuaries of the West Coast. Sampling during this study took place in one of the warmer months of the year and may represent highest levels. As seasonal water temperatures drop during the winter months, levels of these species would be expected to be lower.

No CT producing isolates were observed using the Y-1 mouse adrenal cell tissue test. However, one *V. cholerae* isolate from an oyster sample, Station 5, gave a cytotoxic response in the cell line. The low incidence of strains giving a cytotoxic response and the lack of CT producing strains was also found in our previous work on the West Coast (*7*).

The presence of *V. parahaemolyticus* in all samples was not surprising. Historically, our laboratory has found a high incidence of this halophile in estuaries of the Pacific Northwest (unpublished data). One isolate from an oyster sample (Station 13) was found to be Kanagawa positive, an *in-vitro* measure of the thermostable direct hemolysin (TDH). Colony hybridization results using TDH-3 probe confirmed this isolate to contain a segment of the TDH gene sequence, thus suggesting a potential for human illness. The presence of TDH producing strains in environmental samples has historically been low as was found with our present study.

*V. fluvialis* was isolated from 2 water samples (Station 3 and 5) at the detectable level (0.1/100 ml). This species has recently been implicated in human illness (*14*), but environmental studies are limited.

*V. vulnificus* was not isolated from this estuary. A previous study by our laboratory found only one sample in Washington and only 5.9% of West Coast estuarine samples positive for this pathogen (*8*).

The presence of *Vibrio* species did not correlate with the presence of fecal coliforms nor with the level of fecal coliforms within a sample. Several samples were found to contain *V. cholerae* non-01, while fecal coliforms were not detected. No correlation between prevalence of *Vibrio* species with fecal coliform levels has been reported by others (*4,13*).

The presence of *Vibrio* species in this growing area of commercial oysters was not unexpected due to previous reports from U.S. estuaries and from previous work in our laboratory. Since a cytotoxic non-01 *V. cholorea* and a

TABLE 1. *The frequency of isolation and concentration of* Vibrio *species and fecal coliforms in water, oyster, and sediment samples collected from Grays Harbor.*

| | (n) | VC[1] | VM | VP | VF | VV | FC |
|---|---|---|---|---|---|---|---|
| Water[2] | 18 | 14 | 4 | 18 | 2 | 0 | 17 |
| | | 0.1-40[3] | 0.1-2.3 | 0.1-40 | 0.1 | - | 4-930 |
| Oyster | 23 | 3 | 1 | 23 | 0 | 0 | 14 |
| | | 0.1-0.3 | 0.1 | 0.4-15 | - | - | 36-4600 |
| Sediment | 4 | 0 | 0 | 4 | 0 | 0 | 3 |
| | | - | - | 8-1500 | - | - | 73-230 |
| TOTAL | 45 | 17 | 5 | 45 | 2 | 0 | 34 |

[1]VC = *V. cholerae*, VM = *V. mimicus*, VP = *V. parahaemolyticus*, VF = *V. fluvialis*, VV = *V. vulnificus*, and FC = Fecal Coliform.
[2]Temp. range 13.5 - 18.0°C, Sal. 0.0 - 30.6%.
[3]Reported as the range of MPN per 100 g or ml of sample.

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TDH producing strain of *V. parahaemolyticus* were isolated from harvestable shellstock, a potential for human illness exists. However, low levels of these *Vibrio* species were found during a warm time of year when levels would be expected to be highest. The fact that West Coast commercial shellfish have not been implicated in *V. cholerae* illness and only sporadic cases of *V. parahaemolyticus* illness have been reported in the Northwest by local health departments would indicate a low risk to consumers of oysters harvested from Grays Harbor and other West Coast growing areas, unless some other environmental impact occurs.

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