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Ecology of *Vibrio vulnificus* in Galveston Bay oysters, suspended particulate matter, sediment and seawater: detection by monoclonal antibody – immunoassay – most probable number procedures

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SUMMARY

Oysters, suspended particulate matter (SPM), sediment and seawater samples were collected from West Galveston Bay, Texas over a 16-month period and analyzed for the presence of *Vibrio vulnificus*, a naturally-occurring human marine pathogen. Detection and enumeration of *V. vulnificus* was performed using a species-specific monoclonal antibody (mAb FRBT37) in an enzyme immunoassay (EIA)-most probable number (MPN) procedure capable of detecting as few as 2000 target organisms. *V. vulnificus* was not detected in seawater, oyster or SPM samples during the cold weather months, but was detected at low levels in several sediment samples during this time period. Increased levels of the organism were first observed in early spring in the sediment, and then in SPM and oysters. The major increase in *V. vulnificus* occurred only after the seawater temperature had increased above 20 °C and the winter-spring rainfall had lowered the salinity below 16‰. The highest *V. vulnificus* levels at each site were associated with suspended particulate matter. These results are consistent with the hypothesis that (1) *V. vulnificus* over-winters in a floc zone present at the sediment-water interface, (2) is resuspended into the water column in early spring following changes in climatic conditions, (3) colonizes the surfaces of zooplankton which are also blooming during early spring and (4) are ingested by oysters during their normal feeding process.

INTRODUCTION

The American oyster, *Crassostrea virginica*, is one of the most commercially valuable bivalves in the world [5] and its fishery in the Gulf of Mexico has developed as the primary oyster producer for the nation [1,2]. The oyster fishery is concentrated in the Texas and Louisiana region, and any negative publicity involving that fishery would cause severe economic consequences for the area.

In recent years the association of the bacterium *Vibrio vulnificus* with serious illness and death following raw oyster consumption, has become a major concern for the oyster industry [6,9,14]. During the past 4 years (1987–1990), 13 of 31 (42%) Texas residents with confirmed *V. vulnificus* infections died [12]. Infections increase between April and October and coincide with elevated environmental counts of the bacterium that occur during periods of high water temperature and low salinity [6]. Although most of the serious illnesses and deaths have been associated with individuals having impaired liver functions or compromised immune systems [6,9,14], an overall decreased consumer confidence threatens the Gulf States' oyster industry.

Vibrio vulnificus, first described in 1976 [3], was originally referred to as the 'lactose-positive' vibrio. The bacterium is a widely distributed natural inhabitant in the marine environment [9], and is not associated with high fecal coliform counts or other indicators of pollution as are most other lactose-positive Gram-negative bacteria. Previous techniques used to identify *V. vulnificus* in environmental samples required time-consuming isolation and identification procedures and failed to separate *V. vulnificus* from other bacterial species. Isolation procedures for the identification of *V. vulnificus* typically included incubation of test samples in alkaline peptone water (APW), followed by isolation on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Subsequent identification was performed using a battery of biochemical and growth tests [10]. These methods are complicated by the replication of

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other bacteria in APW as well as in other media. This means that numerous growth cultures must be tested through a series of biochemical assays that are costly and time consuming. Because *V. vulnificus'* presence is not linked to total coliform numbers, other methodologies for its identification had to be developed.

The task of formulating new isolation media was undertaken by Oliver and Massad and resulted in the development of cellobiose-polymyxin B-colistin agar (CPC) [7]. CPC agar utilizes the antibiotic resistance of *V. vulnificus* and *Vibrio cholerae* toward colistin and polymyxin B. This agar in conjunction with an incubation temperature of 40 °C, which eliminates most other marine bacteria, and *V. vulnificus'* ability to ferment cellobiose at this temperature makes the agar presumptively differential for *V. vulnificus* [7]. However, at least one strain of *Vibrio parahaemolyticus* is also a cellobiose fermenter at this temperature. Few field studies have been performed using CPC agar, but it has proven to be an effective means of narrowing down the environmental isolates.

Recently Tamplin et al. developed a monoclonal antibody (mAb FRBT37) to a species-specific epitope of *V. vulnificus* to be used in an enzyme immunoassay (EIA) for the detection of relatively low numbers (2000 cell minimum detection) of *V. vulnificus* [11]. The antibody was purified from mAb FRBT37 ascites fluid using protein A-Sepharose, and tested against 72 non-*V. vulnificus* strains comprising 34 species and 15 genera, resulting in no cross-reactions. Field trials showed a 99.7% correct identification of 348 biochemically confirmed *V. vulnificus* isolates [11]. This EIA, along with the use of CPC agar and an MPN procedure, forms the basis of the detection and enumeration techniques utilized in this study. The current field study was conducted to determine the ability of the combined techniques to detect and enumerate *V. vulnificus* in its natural habitat over an extended period of time.

MATERIALS AND METHODS

Sample sites. Three oyster reef sites in the Galveston Bay complex were chosen for testing during this study. The first site, Sammy's Reef, is a young, man-made reef, containing a soft bottom, and located away from the main body of the bay. The soft silty substrate is a result of the slow water currents that flow over the reef, which allow deposition of suspended material. The second, Deer Island Reef, is an old commercial reef, in the main body of West Bay, that is now closed due to pollution from nearby industry. The third, Confederate Reef, is an active commercial oyster reef located in the central body of the West Galveston Bay system. Water depths at all three sites range from a fully exposed reef to approximately one meter in depth depending on the tides. Oyster, sediment, suspended particulate matter (SPM) and seawater samples were collected at each site, along with temperature and salinity measurements. Temperature was determined with a calibrated field thermometer and salinity with a hand-held field refractometer.

Sample collection. Water samples were collected in a sterile bottle and 1.0-ml portions were used in a most probable number (MPN) enumeration procedure. Sediment samples, aseptically collected from each site, were returned to the lab, the excess water was poured off and 10 g (wet weight) of each sample was mixed with 10 ml of phosphate buffered saline (PBS) (pH 7.4). The resultant slurry, in 2.0-ml aliquots, was used for the MPN procedure. Sufficient oysters were collected from the field sites to yield 100 g of meat after cleaning and shucking. To each 100-g sample of oyster meats, 100 ml of PBS was added and blended in a sterile stainless steel container for 90 s. Two-ml aliquots of the oyster homogenate were then used in the MPN procedure. The SPM samples were taken by walk-towing a #25 plankton net for 1 min. A General Oceanics flow meter (model No. 2030) was suspended in the mouth of the plankton net in order to determine the volume of seawater sampled. The concentrated seawater was collected in a 450-ml container at the back end of the plankton net and 1.0-ml aliquots were used for the MPN analysis. The MPN values for the SPM samples were corrected for the concentration effects of the plankton net, and then normalized by standardizing the value to 1 g. In order to accomplish this a concentration factor (C.F.) for each sample was derived from the volume of seawater filtered through the plankton net, and the dry weight of the sample determined. The concentration factor was obtained by dividing the volume of seawater filtered by the volume of concentrated sample (450 ml). In order to determine the dry weight of SPM, 10.0-ml aliquots of each sample were dried at 70 °C for 48 h and weighed until a stable value was reached. The resultant weight was then divided by 10 to give a gram weight for 1.0 ml concentrated SPM. That number was then divided by the C.F. to give a weight of SPM per ml sample of unconcentrated seawater.

Enumeration of V. vulnificus. The levels of *V. vulnificus* in all samples were determined using a most probable number (MPN) procedure, as outlined in Tamplin et al. [11]. The samples were serially diluted to 1×10^{-7} in PBS, each dilution was inoculated into three tubes, each containing 10 ml of alkaline peptone water (APW). The tubes were incubated at 37 °C for 12–16 h. A loopful from each APW tube was then streaked onto CPC agar plates, and incubated for 24 h at 40 °C to diminish the growth of unwanted endogenous bacteria. Yellow colonies that developed on the CPC agar, usually translucent colonies

with an opaque center, were transferred to 96-well tissue culture plates containing 100 μl of APW per well. After 6–8 h incubation at 37 °C, 25-μl aliquots of the APW cultures were transferred to each of two 96-well EIA plates. One plate was used for the assay and the other as a negative control. *V. vulnificus* (UT290) was the positive control. After transferring the cultures, 25 μl of Triton X-100 (0.02% solution) was added to the wells, as a lysing and coating solution, and the plates were dried overnight in a 37 °C incubator.

Each dried well received 200 μl of 1% bovine serum albumin (BSA) to block or mask non-specific binding sites and incubated for 1 h at room temperature. The plates were then washed and 50 μl of the *V. vulnificus*-specific monoclonal antibody (diluted 1/4 with PBS) was added to the wells of one plate. The wells of the negative control plate were filled with 50 μl of PBS. After a 1-h, room temperature incubation the plates were washed and treated with 50 μl (diluted 1/500 with PBS) of peroxidase-conjugated goat anti-mouse IgG (Sigma Cat. no. A3673) and incubated for 1 h in the dark at room temperature. The plates were then rewashed and treated with 100 μl of a 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) substrate solution (1% ABTS in 0.05 M citric acid, pH 4.0). After 10–15 min the wells containing *V. vulnificus* turned green. The MPN of *V. vulnificus* was then determined, relating each positive well back to its original APW tube.

RESULTS AND DISCUSSION

Physical parameters

Environmental samples as well as salinity and temperature measurements were collected from March 1990 through June 1991 and analyzed for the presence of *V. vulnificus*. Since all three sample sites yielded similar results and trends, only the data for Confederate Reef are presented. Seawater temperature was lowest in early winter (January), increased to a peak in early summer, and remained high until early fall. The seawater temperature ranged from a low of 12 °C in January 1991 to a high of 31 °C in June 1990 (Fig. 1). Salinity followed an almost opposite trend, decreasing with rainfall which was heaviest during the winter/early spring months (low salinity), and increasing with the diminished precipitation [8] of the summer and fall months. Salinity ranged from a high of 30‰ to a low of 10‰ (Fig. 1).

Enumeration of V. vulnificus

The levels of *V. vulnificus* (MPN/g) in SPM samples started to increase in early spring and continued to rise though early summer, after which they began to decline (Fig. 2A–D). From early fall until the following spring, SPM *V. vulnificus* levels remained low. MPN values ranged from <0.1/g to 5.5×10^7/g; even the uncorrected data yielding a high of 1.1×10^6/g (Fig. 2A). Sediment samples followed the same trend as that observed for the SPM samples. Sediment bacterial counts ranged from a high of 2.1×10^5/ml to a low of <0.1 ml (Fig. 2B). Oyster samples followed the same rise and decline pattern, ranging from a high of 1.2×10^3/ml to a low of <0.1/ml (Fig. 2C). MPN values for seawater samples displayed the same trends as those observed for the other sample types, but with lower levels of *V. vulnificus* detected. Seawater MPNs ranged from 2.1×10^3/ml to <0.1/ml (Fig. 2D).

V. vulnificus was recovered in low concentrations in all sample types from September to December. Between January and March the organism was detected only in isolated sediment samples, during which time salinity remained above 18‰ and the temperature was below 20 °C. Once physical conditions became more suitable for the bacterium's growth (i.e., <16‰ salinity and >20 °C) the MPN for all sample types began to increase. For the first few weeks, *V. vulnificus* levels were highest in sediment samples, but then SPM levels began increasing dramatically. Bacterial counts on SPM were consistently the highest among all the sample types, followed by sediment, oyster and then seawater.

Since *V. vulnificus* was detected in sediment samples only during the winter months and increased MPN levels are first associated with sediments, the organism may be 'over-wintering' in or near the bottom sediments. *V. vulnificus* may be inhabiting a thin floc zone directly above the sediment [15]. Initial growth of the bacterium coincides with the rainy season [8] during which run-off, rainfall, and accompanying high winds mix the shallow bay waters. If the bacterium is colonizing a floc zone just above the sediment-water interface during the winter, it could readily enter the water column following these perturbations. This could explain *V. vulnificus* levels in SPM equal to that of the sediment samples, but not the marked increase that was observed in the concentrated particulate fraction.

In the water column, the bacteria are exposed to nutrients that are washing down the rivers or banks due to heavy rainfall, and they became resuspended by wave action. Additionally, a particular nutrient may induce and support growth in SPM. During the early spring months there is usually a phytoplankton bloom in Galveston Bay followed approximately a month later by a zooplankton bloom [13]. *V. vulnificus* can utilize chitin as a food source and since this material makes up the exoskeleton of zooplankton, the bacteria could readily colonize the chitinous exoskeleton of the zooplankton and be detected in increased numbers in the SPM. This colonization effectively provides ample surface area upon which the bac-

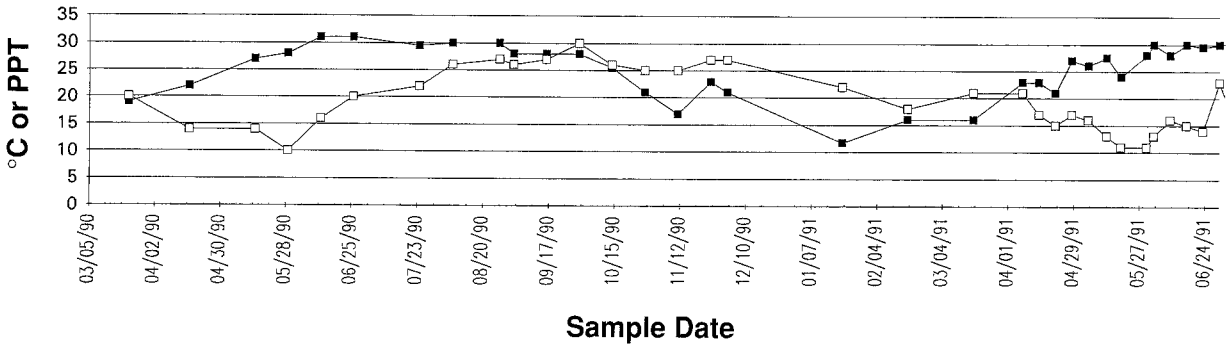

Fig. 1. Temperature and salinity measurements recorded for Confederate Reef. ■, temperature in °C; □, salinity in parts per thousand (PPT).

teria can multiply, and simultaneously offers a food source for the bacterium to use, effectively increasing the numbers of the bacterium above that found in the sediment samples [4].

These results are consistent with the hypothesis that (1) *V. vulnificus* over-winters in a floc zone present at the sediment-water interface, (2) is suspended into the water column as SPM in early spring by changes in climatic

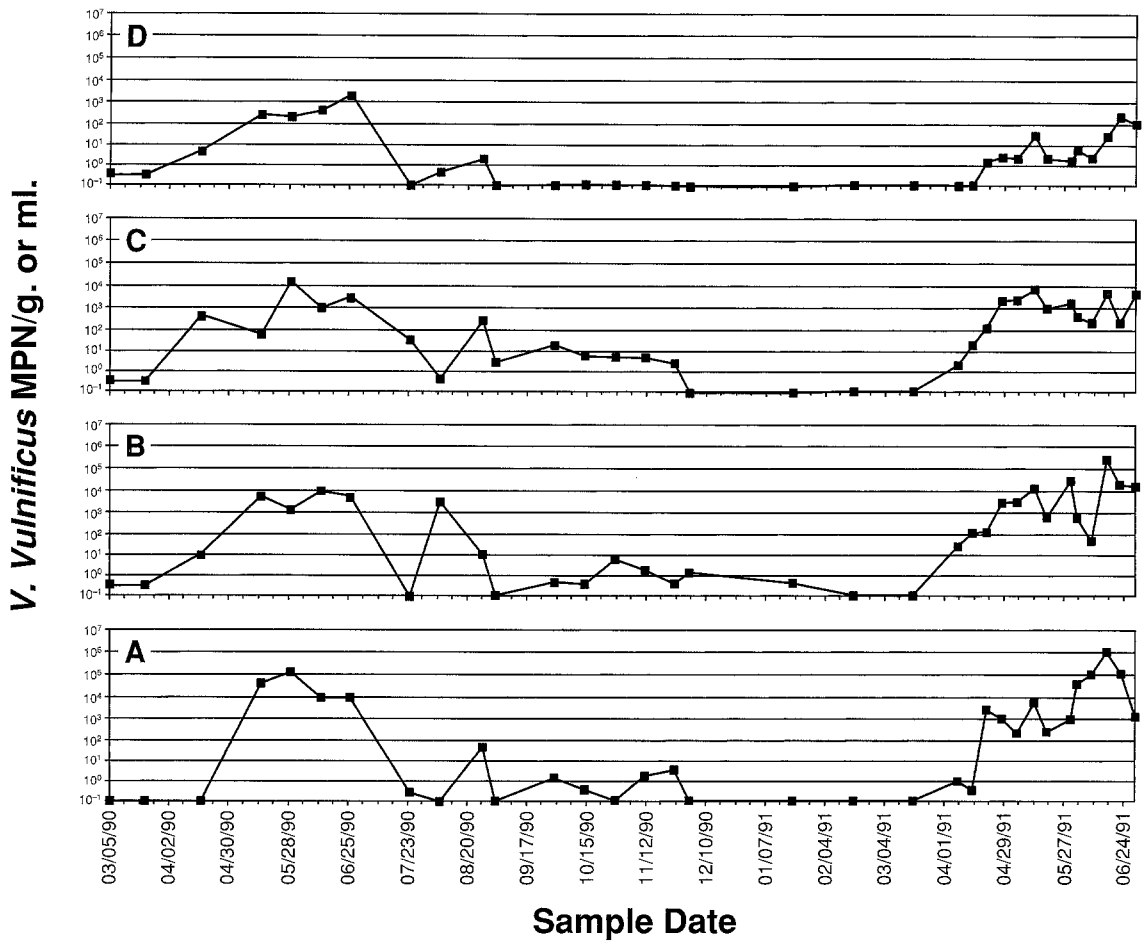

Fig. 2. Recovery of naturally-occurring *Vibrio vulnificus* from different Confederate Reef sample types. (A) Suspended particulate matter (SPM), expressed as most probable number (MPN) per ml of concentrated sample. (B) Sediment, expressed as MPN per g wet weight of sample. (C) Oysters, expressed as MPN per g of oyster meat. (D) Seawater, expressed as MPN per ml of seawater.

conditions, (3) colonizes the surfaces of zooplankton that are blooming during April–May and (4) are ingested by oysters during their normal feeding process. Studies are under way to differentiate the sequence of events accompanying the growth cycle of *V. vulnificus* in its natural habitat.

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