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# Densities of *Vibrio vulnificus* in the Intestines of Fish from the U.S. Gulf Coast

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**Densities of *Vibrio vulnificus* in the intestinal contents of various finfish, oysters, and crabs and in sediment and waters of the U.S. Gulf Coast were determined by the most probable number procedure. Species were identified by enzyme immunoassay. During the winter, densities of *V. vulnificus* were low, and the organism was isolated more frequently from sheepshead fish than from sediment and seawater. From April to October, *V. vulnificus* densities were considerably higher (2 to 5 logs) in estuarine fish than in surrounding water, sediment, or nearby oysters and crustacea. Highest densities were found in the intestinal contents of certain bottom-feeding fish ($10^8$/100 g), particularly those that consume mollusks and crustaceans. Densities of *V. vulnificus* in fish that feed primarily on plankton and other finfish were similar to those in oysters, sediment, and crabs ($10^5$/100 g). *V. vulnificus* was found infrequently in offshore fish. The presence of high densities of *V. vulnificus* in the intestines of common estuarine fish may have both ecological (growth and transport) and public health (food and wound infections) implications.**

*Vibrio vulnificus*, a virulent pathogen (11, 20, 28) that is prevalent in estuaries of the U.S. Gulf Coast, is transmitted to humans through wounds or from the consumption of food, usually raw oysters (3, 12). Symptoms of food-borne illnesses include gastroenteritis and primary septicemia. Although the number of primary septicemia cases is low, the fatality rate is high (approximately 50%) in immunocompromised individuals and in the chronically ill, particularly those with underlying liver disease.

*V. vulnificus* has been isolated from the Atlantic, Pacific, and Gulf coasts of the United States and is present in Gulf Coast estuaries (10, 11, 16–18, 20, 28); densities are greatest when the water temperature is high. Most environmental studies of vibrios have focused on their presence in molluscan shellfish, crustacea, plankton, seawater, and sediment. However, the lack of quantitative data in most of these investigations has impeded progress toward determining sources or niches of these vibrios. Recently, isolation and identification of *V. vulnificus* have been improved by the use of cellobiose-polymyxin B-colistin agar (14) and enzyme immunoassay (EIA) (27). Similar procedures were used in an environmental survey of waters and oysters in the Great Bay estuary of Maine and New Hampshire (18).

Vibrios have frequently been isolated in high densities from the intestines of cultured and wild finfish from Japan, Europe, and the United States (5, 9, 13, 15, 21–23, 29–31) and were found to be the dominant microflora of marine fish with well-developed digestive systems (23). In contrast with most other bacteria in the fish diet (24), these vibrios, not identified to species level, were not affected by stomach acidity (pH 4). Although *V. vulnificus* has been found in finfish of the U.S. Atlantic Coast, data on its densities, its anatomical location, and the fish species were not reported (16, 17). A variety of finfish, frequently in large schools, inhabit the oyster reefs along the U.S. Gulf Coast. These fish feed on plankton, crustacea, and mollusks, which are known reservoirs of *V. vulnificus* (16, 17, 26). High nutrient levels in the fish intestine also may enhance the growth of *V. vulnificus*. The purpose of this study was to determine whether common estuarine fish of the U.S. Gulf Coast may, therefore, be a substantial source of *V. vulnificus*.

## MATERIALS AND METHODS

**Sample collection and preparation.** Fish were collected from Alabama waters, including Mobile Bay and Mississippi Sound, and from waters of the Gulf of Mexico up to 30 mi (ca. 60 km) offshore. Fish were caught with hook and line, cast nets, gill nets, trawls, traps, and spears. Individual fish were placed in plastic bags and iced unless analysis was initiated within 1 h of harvest. All fish were examined within 4 h of harvest. The external abdominal surface of the fish was swabbed with 70% ethanol to reduce potential contamination of the intestinal contents with skin bacteria. An incision was made over the peritoneal cavity; the intestines were severed slightly anterior to the pyloric valve and anus, and the contents of the intestine were aseptically massaged into a sterile petri dish and mixed by stirring with a sterile pipette. The intestinal contents of small fish or those with nearly empty intestines were pooled to obtain enough material for analysis. Stomach contents of some fish were massaged from the severed esophagus. Intestine and stomach contents were examined visually for shell and bone fragments to determine diet.

Water, oyster, and sediment were collected along with the fish. Surface water at fish harvesting sites was collected aseptically in sterile 500-ml wide-mouth polyethylene bottles, according to procedures of the American Public Health Association (1). Oysters (10 to 12) were collected with oyster tongs or by dredging and were scrubbed and shucked according to American Public Health Association procedures (1). Blue crabs (one to three) were collected with traps, and mud crabs (three to five) were removed from oyster shells and rinsed with overlying surface waters to remove mud. Sediment was also collected with oyster tongs, and the top 1-cm layer was placed

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TABLE 1. Distribution of *V. vulnificus* in water, sediment, and oysters and the intestinal contents of the sheepshead fish with time

| Date collected (mo/day/yr) | Water temp (°C) | Salinity (‰) | No. of fish analyzed | *V. vulnificus* MPN/100 g or ml ($log_{10}$) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Fish[a] | Water | Sediment | Oyster |
| 1/23/91 | 11 | 13 | 1 | 6.0 | 2.0 | 2.9 | 3.0 |
| 2/04/91 | 13 | 9 | 10 | 1.6 | <1.5 | ND[b] | <1.5 |
| 2/22/91 | 13 | 15 | 5 | 2.1 | <1.5 | <1.5 | <1.5 |
| 6/21/91 | ND | ND | 1 | 8.6 | ND | ND | ND |
| 7/02/91 | 30 | 5 | 1 | 9.1 | 3.4 | 5.6 | 7.0 |
| 1/13/91 | 11 | 22 | 5 | <1.5 | <1/5 | <1/5 | <1/5 |
| 1/27/92 | 11 | 8 | 5 | <1.5 | <1.5 | <1.5 | <1.5 |
| 4/01/92 | 11 | 18 | 5 | 2.4 | <1/5 | 2/2 | 2.4 |
| 4/23/92 | 23 | 20 | 5 | 7.9 | 3.4 | 5.0 | 4.4 |
| 6/22/92 | ND | ND | 2 | 7.9 | 3.4 | 5.6 | 7.4 |
| 7/13/92 | 30 | 20 | 2 | 8.3 | 3.4 | 5.2 | 5.2 |

[a] Mean density in sheepshead intestinal contents.
[b] ND, not determined.

aseptically in 50-ml polypropylene tubes. Animal samples were placed in plastic bags and, along with water and sediment samples, were transported in an ice chest with bagged ice to the laboratory. All samples were analyzed within 4 h of collection.

**Bacteriological analyses.** Specimens were examined by procedures described in the Bacteriological Analytical Manual of the Food and Drug Administration (8) and adapted for most probable number (MPN) estimates. Whole live crabs and oyster meats were diluted in sufficient volumes of alkaline peptone water (APW) or phosphate-buffered saline (PBS), 1:1 to 1:5, to facilitate pipetting and blended for 1.5 min in a Waring blender at 14,000 rpm. Fish intestinal contents, sediment, and water were mixed by vigorous shaking in tubes or bottles of diluents. Serial 10-fold dilutions were prepared in PBS. A three-tube MPN series consisting of screw-cap tubes (16 by 150 mm) with 10 ml of APW was inoculated, beginning with 1.0-g portions (0.1 g for fish with <7.0 g of intestinal contents) and ending with $10^{-9}$ g.

An MPN procedure using 96-well culture plates (beginning with $10^{-2}$-g portions in 200 μl of APW) was substituted for the MPN tube procedure to facilitate analysis of specimens with suspected high vibrio densities. Serial 10-fold dilutions were prepared by transferring 20 μl into successive wells of a sterile 96-well culture plate (no. 3593; Costar, Cambridge, Mass.) containing 180 μl of PBS. A new tip was used for each transfer, and the well contents were mixed by aspirating 20 times with the micropipettor. Aliquots (20 μl) were transferred with a multichannel pipettor with seven pipette tips into wells containing 180 μl of APW (three wells per dilution).

APW tubes or 96-well culture plates were incubated overnight at 35°C. Turbid APW was streaked to cellobiose-polymyxin B-colistin agar (14) modified to contain 400,000 U of colistin methanesulfonate per liter (27) and incubated overnight at 40°C. Typical colonies (two per tube or well) were presumptively identified as *V. vulnificus* by EIA (27). *V. vulnificus* suspects were identified by the API 20E system (Analytab Products, Inc., Plainview, N.Y.).

## RESULTS

Table 1 shows the distribution of *V. vulnificus* in the intestinal contents of the sheepshead fish and in water, sediment, and oysters with time. Samples were collected at about the same time (within 1 h) and location (within 30 m). Densities were highest in spring and summer. When *V. vulnificus* was abundant, densities in sheepshead intestines were generally 2 to 4 logs greater than in corresponding oyster and sediment and approximately 5 logs higher than in seawater. In specimens collected in February, *V. vulnificus* was found in sheepshead intestines on two occasions when it was not detected in other specimens. On two other occasions, it was not found in any of the specimens.

Table 2 lists the densities of *V. vulnificus* in the intestinal contents of various fish and in environmental specimens collected from inshore estuarine waters during the spring and summer. Bottom fish such as sheepshead, pigfish, black drum,

TABLE 2. Density of *V. vulnificus* in water, sediment, oysters, and crabs and the intestinal contents of fish collected during the months of April through October from Mobile Bay and the Mississippi Sound in Alabama

| Sample type | No. of samples | Mean *V. vulnificus* MPN/100 g or ml ($log_{10}$) | SD |
|---|---|---|---|
| Water | 9 | 2.9 | 0.7 |
| Sediment | 8 | 5.6 | 0.8 |
| Oyster (*Crassostrea virginica*) | 7 | 5.2 | 1.3 |
| Blue crab (*Callinectes sapidus*) | 3 | 4.8 | 0.4 |
| Mud crab (*Panopeus* spp.) | 4 | 5.0 | 1.2 |
| Atlantic croaker (*Micropogonias undulatus*) | 3 | 7.8 | 0.6 |
| Atlantic spadefish (*Chaetodipterus faber*) | 1 | 3.4 | |
| Atlantic stingray (*Dasyatis sabina*) | 1 | 4.6 | |
| Black drum (*Pogonias cromis*) | 8 | 8.5 | 0.7 |
| Crevalle jack (*Caranx hippos*) | 5 | 4.5 | 2.6 |
| Gafftopsail catfish (*Bagre marinus*) | 1 | >1.5 | |
| Gulf menhaden (*Brevoortia patronus*) | 4 | 5.4 | 1.8 |
| Gulf toadfish (*Opsanus beta*) | 1 | 8.4 | |
| Pigfish (*Orthopristis chrysoptera*) | 8 | 8.8 | 1.1 |
| Pinfish (*Lagodon rhombodies*) | 3 | 8.5 | 0.3 |
| Red drum (*Sciaenops ocellatus*) | 3 | 2.5 | 1.4 |
| Scaled sardine (*Harengula jaguana*) | 1 | 5.4 | |
| Sea catfish (*Arius felis*) | 8 | 8.8 | 1.3 |
| Sheepshead (*Archosargus probatocephalus*) | 11 | 8.2 | 1.0 |
| Southern kingfish (*Menticirrhus littoralis*) | 1 | 5.4 | |
| Spanish mackerel (*Scomberomerus maculatus*) | 3 | 5.5 | 1.0 |
| Striped mullet (*Mugil curema*) | 5 | 4.8 | 2.7 |

TABLE 3. Recovery of *V. vulnificus* from the intestinal contents of offshore fish from the Gulf of Mexico in July and August 1992

| Species | No. positive for *V. vulnificus*/no. of samples |
|---|---|
| Atlantic spadefish (*Chaetodipterus faber*) | 0/2 |
| Cobia (*Rachycentron canadus*) | 0/2 |
| Gafftopsail catfish (*Bagre marinus*) | 0/1 |
| Greater amberjack (*Seriola dumerili*) | 0/1 |
| Little tuna (*Euthynnus alletteratus*) | 1/2 |
| King mackerel (*Scomberomerus cavalla*) | 0/2 |
| Red snapper (*Lutjanus campechanus*) | 1/5 |
| Spanish mackerel (*Scomberomerus maculatus*) | 0/1 |
| Vermillion snapper (*Rhomboplites aurorubens*) | 0/1 |

sea catfish, pinfish, spot, Atlantic croaker, and Gulf toadfish contained the highest densities of *V. vulnificus*. Shells of crustacea and mollusks were often present in the intestinal contents of sheepshead, black drum, pigfish, and pinfish. Densities of *V. vulnificus* in planktivores (Gulf menhaden, scaled sardine), herbivores (striped mullet), and carnivores (crevalle jack and Spanish mackerel) were lower than those found in bottom fish and similar to those in sediment, oysters, and crabs. Low densities were also found in red drum, which usually feed on either crustacea or finfish (2). These were collected from the higher-salinity waters of Petis Bois Pass, which separates the Mississippi Sound from the Gulf of Mexico.

During February (water temperature, 13°C) *V. vulnificus* was found in the intestinal (75%) and stomach (38%) contents of eight sheepshead; mean densities were 2.0 and 1.6 ($\log_{10}$) per 100 g, respectively. During the summer (water temperature, 30°C), *V. vulnificus* densities in the stomach and intestinal contents of a pigfish were 7.0 and 8.6 ($\log_{10}$) per 100 g, respectively, and 5.4 and 8.0, respectively, in a sea catfish.

*V. vulnificus* was found in only two fish (one little tuna and one red snapper) collected from offshore sites in the Gulf of Mexico (Table 3). The *V. vulnificus* densities were approximately $10^5$/100 g of intestinal contents in both fish. The little tuna was collected next to a natural gas production platform, and the red snapper was collected on a berm of dredge spoil from Mobile Bay channel, each site approximately 5 km offshore.

A total of 1,924 isolates were selected from cellobiose-polymyxin B-colistin, and 1,494 (78%) of these were positive by EIA. However, less than 10% (9/93) of isolates from fish collected at offshore sites were positive by EIA. Of 44 isolates presumptively identified as *V. vulnificus* by EIA and tested in the API 20E system, 42 (95%) were confirmed as *V. vulnificus*. The remaining two isolates, from sheepshead intestinal contents, were identified as *Vibrio alginolyticus*.

## DISCUSSION

*V. vulnificus* is abundant in a variety of estuarine fish species that commonly inhabit oyster reefs of the U.S. Gulf Coast. Vibrios have been identified worldwide as a major component of the intestinal microflora of both cultured and wild fish (5, 9, 13, 15, 21–23, 29–31); however, the presence of *V. vulnificus* in finfish has received little attention. In two related studies (16, 17), environmental specimens from the U.S. Atlantic Coast were examined for lactose-fermenting marine vibrios, and *V. vulnificus* was recovered from finfish, crabs, and other invertebrates. In those studies, the total vibrio counts in non-filter-feeding animals (two crabs and a fish not identified to species level) were lower than those in oysters. In the present study, *V. vulnificus* densities in the intestines of some fish were considerably higher than those in surrounding water and sediments or in nearby crabs and oysters, suggesting that fish are important in the ecology of *V. vulnificus*.

The distribution of *V. vulnificus* was studied in the intestines of sheepshead, which is abundant year-round on the U.S. Gulf Coast (2). Higher densities of *V. vulnificus* were found in the sheepshead intestine than in corresponding water, sediment, and oysters. The density of *V. vulnificus* in the oyster gastrointestinal contents was not determined; however, this organism is numerous in other oyster tissues (25). The finding that densities were low in winter and high in summer was in agreement with results of previous environmental studies (11, 17, 20). The observation of *V. vulnificus* in the sheepshead intestine but not in environmental specimens suggests that the organism may overwinter in these fish. Although there was limited growth (<1.0 log) in the intestine during the winter, i.e., densities were higher than those in the stomach, at certain times during the winter, *V. vulnificus* was not found in any specimens.

In summer, when *V. vulnificus* is most prevalent, fish were collected by various techniques to obtain specimens representing different niches and feeding habits, with the primary focus on oyster reefs. High densities of *V. vulnificus* were usually found in intestinal contents that contained fragments of mollusk shells and crustacean exoskeleton (e.g., sheepshead and black drum). Small fragments consisting of oyster (*Crassostrea virginica*), ribbed mussel (*Ischadium demissum*), barnacle (undetermined species), and mud crab shells (*Xanthidae* spp.) were prevalent in the intestines of most sheepshead, which is consistent with their reported feeding behavior (2). Clam (*Rangia cuneata*) shell fragments were prevalent in the larger black drum (>10 kg), whereas mud crabs and mussel shells were found in the smaller drum (1 to 2 kg). Crab and mussel shell fragments were found in the intestines of some pigfish, pinfish, and sea catfish. The feeding behavior of these fish can vary, but they are primarily bottom feeders, which eat small fish, shrimp, crabs, benthic worms, and organic detritus (2, 7, 19). *V. vulnificus* was less numerous in the planktivores (Gulf menhaden and scaled sardines) and carnivores such as crevalle jacks and Spanish mackerel, which frequently feed on planktivores. *V. vulnificus* was associated primarily with benthic species rather than the planktonic species of Mobile Bay (26), and its numbers varied most in the striped mullet, which feeds on plants and organic detritus (2).

Geographical location apparently influenced the prevalence of *V. vulnificus*, which was isolated infrequently (11.8%) from fish collected offshore in the Gulf of Mexico. Gulf waters were generally more saline (32 to 35‰ NaCl) than inshore sites (5 to 28‰ NaCl) where *V. vulnificus* was prevalent. *V. vulnificus* was isolated less frequently (13%) from the more saline (18.9‰ NaCl) open Gulf beaches of Galveston, Tex., than in the less saline (11.3‰ NaCl) sites in the Galveston Bay estuary where it was recovered from 68% of the specimens (11).

Many fish species inhabit both estuarine and offshore waters. In the present study, *V. vulnificus* was found in Spanish mackerel and Atlantic spadefish from inshore areas but not in those caught offshore (5 to 50 km). *V. vulnificus* may have been found in the intestines of a red snapper (one of five) and little tuna (one of two) because these fish feed on small fish or shrimp that migrate from estuaries into the Gulf. Tidal flow from Mobile Bay may have been the source of these organisms, but *V. vulnificus* densities in the Bay waters were lower than those in the intestines of these fish, and the distance (>5 km

through deeper open Gulf waters) separating the fish collection sites from the mouth of Mobile Bay would result in considerable dilution.

Intestinal multiplication may account for the higher densities of *V. vulnificus* in the intestine than in the stomach contents of a pigfish and a sea catfish caught in a gill net. *V. vulnificus* multiplies in the tissues of live oysters in aquaria with individual oysters, releasing $10^5$ to $10^6$ organisms per h (25). Most of the fish were collected by hook and line, and the presence of bait in the stomach prevented valid comparison of stomach and intestinal contents. More controlled studies are required to determine the extent of bacterial growth in various parts of the fish digestive tract. Attachment or colonization studies would also provide insight into the ecology of *V. vulnificus* in the fish intestine but were beyond the scope of this study.

The combination of cellobiose-polymyxin B-colistin agar for isolation (14) and species-specific monoclonal antibody-based EIA procedure for identification of *V. vulnificus* (27) used in this study was efficient and highly specific for this organism. Over 95% of the presumptive isolates were confirmed as *V. vulnificus* when tested in the API 20E system. The fact that these techniques were not available during many previous environmental surveys for *V. vulnificus* (10, 11, 16, 17, 28) may compromise data comparison with the present study. The quantitative data obtained in this study were essential in evaluating potential sources and growth of *V. vulnificus* at various times, locations, and estuarine niches. Use of 96-well culture plates and multichannel micropipettors facilitated the MPN procedure by reducing the time of medium preparation, dilution of test material, inoculation, and streaking of enrichment broth for isolation. The sensitivity of this method is 30 cells per g unless it is used in combination with tubes for lower dilutions containing 1.0- and 0.1-g portions.

In a study of the Great Bay estuary of Maine and New Hampshire, mean *V. vulnificus* densities per 100 g or ml ($\log_{10}$) in paired oyster and water collected from July to October were 4.1 and 2.3, respectively (18). These densities are approximately 1 log lower than those observed in oyster and seawater in the present study, probably because of the cooler climate of that region. *V. vulnificus* densities frequently exceeded $10^7$/100 g in commercial shipments of Louisiana oysters harvested from May to September (20). However, the potential for postharvest multiplication of *V. vulnificus* in shellstock oysters may have contributed to the high density of vibrios seen in these oysters (6).

The presence of high densities of *V. vulnificus* in the intestinal contents of fish observed in this study has ecological and public health implications. Unlike filter-feeding bivalves such as oysters, finfish are quite mobile. Thus, there is considerable potential for them to transport this bacterium upcurrent against prevailing winds or tidal flow and possibly over long distances during migrations. Fish harboring high densities of *V. vulnificus* are among the most abundant species in most Gulf Coast estuaries, and they frequently travel in large schools. Their arrival could have an immediate and substantial effect on the water quality and the bacterial content of filter-feeding mollusks, especially in shallow areas with little tidal movement.

Finfish have not generally been associated with *V. vulnificus* infection in humans. In the United States, finfish are usually cooked, and thus, any bacteria present are destroyed. However, lightly cooked and raw fish are gaining popularity, especially among certain ethnic groups.

Like other gram-negative organisms, *V. vulnificus* may migrate from the intestine to the edible portions in living fish (*Escherichia coli*, *Citrobacter freundii*, and *Salmonella* spp. were found in the blood and muscle of carp [*Cyprinus carpio*] and tilapia [*Tilapia aurea*] within 2 h of inoculation into the stomach through an esophageal capillary cannula [4]). Severing the intestines during filleting or eviscerating fish could also contaminate the edible portions with *V. vulnificus* and should be avoided.

The sharp spines of sea catfish, pinfish, and sheepshead can inflict deep wounds. Although the surfaces of fish were not examined in this study, *V. vulnificus* could be introduced into a wound by contact with a fish spine contaminated with fecal material. Immunocompromised and chronically ill individuals who handle these fish, e.g., recreational and commercial fishermen or seafood processing plant workers, would therefore be at risk from such injuries.

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