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Virulent Strains of *Vibrio vulnificus* Isolated from Estuaries of the United States West Coast

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***Vibrio vulnificus* was isolated from United States West Coast estuaries at a low frequency (5.9%) from 529 samples of water, shellfish, and sediment. Four strains tested with iron-treated mice had 50% lethal dose values ranging from 7.6 to 360 CFU, compared with a 50% lethal dose of 4.9 CFU for a clinical isolate that caused the death of a septicemic patient. The presence of this pathogen may be a hazard to users of marine beaches and consumers of raw shellfish on the West Coast, especially to persons most susceptible to *V. vulnificus* septicemia. Species-specific antiflagellar serum and a gene probe for cytotoxin-hemolysin production were useful for screening these environmental isolates.**

A halophilic, lactose-fermenting *Vibrio* species, first described in 1976 as causing septicemia in a human patient (8), was subsequently designated *Vibrio vulnificus* (3). This species causes two distinct clinical syndromes in infected humans, depending on the infection mode (1, 15). Wound infections have occurred in healthy individuals exposed to marine environments. Ingestion of contaminated raw oysters is most frequently associated with primary septicemia, which is characterized by rapid development of sepsis, secondary skin lesions, and a mortality rate exceeding 50%. Most cases resulting in fatalities have involved patients with prior liver dysfunction resulting in high serum iron levels. The hazards associated with ingestion of seafoods contaminated with this pathogen have resulted in a precautionary statement issued by the Food and Drug Administration against consumption of raw or undercooked seafoods by people with hepatic disorders (5).

The role of virulence factors of *V. vulnificus* in the pathogenesis of human infections is not clear. Factors reportedly linked to virulence include siderophore production (19), elevated serum iron levels (25), production of cytotoxic and cytolytic extracellular factors (6, 13), and resistance of strains to the antibacterial activity of human serum (2).

The incidence of *V. vulnificus* is well documented for Atlantic and Gulf Coasts of the United States (12, 16, 20), where most infections have been reported, and for the Oregon coast (23). Two recent oyster-associated infections in California (D. Alton, Food and Drug Administration, personal communication, 1984, 1985) and one in Utah (9) were attributed to oysters not harvested from the West Coast.

The purpose of this study was to determine the incidence and virulence of *V. vulnificus* in marine environments of Washington, Oregon, and California in conjunction with an incidence study of *Vibrio cholerae* (11). The uses of species-specific anti-*V. vulnificus* flagellar sera and DNA colony hybridization techniques for screening *V. vulnificus* were also investigated.

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MATERIALS AND METHODS

Sample collection and handling. During 1984, 529 water, sediment, and shellfish samples were qualitatively examined for *V. vulnificus*. Sample locations are shown in a related study (11). Shellfish included the following species: Pacific oyster (*Crassostrea gigas*), mussel (*Mytilus californicus*, *M. edulis*), gaper clam (*Schizothaerus nuttali*), and cockle (*Clinocardium nuttali*).

The specifics of sample collection are reported elsewhere (11).

Isolation and identification. Samples were enriched in alkaline peptone water (4) and incubated overnight at 35°C. The surface pellicle was streaked to thiosulfate-citrate-bile-salts (Oxoid-USA, Columbia, Md.) and polymyxin-mannose-tellurite (Nissiui, Tokyo, Japan) selective agars. Plates were incubated for 18 to 24 h at 35°C. Suspect colonies were picked and maintained in motility test medium (Difco Laboratories, Detroit, Mich.) with 0.5% added NaCl and screened using an arginine-based medium (10). Identification of isolates followed the scheme of Tison et al. (22). All sucrose-negative isolates were tested for an *o*-nitrophenyl-β-D-galactopyranoside reaction (17) as recommended by Oliver et al. (16).

Reference strains. Reference strains used in the study were *V. vulnificus* LA M6-24 and 85A05667, both human blood isolates, obtained from R. Murray, Microbial Diseases Laboratory, California State Department of Health Services, Berkeley, and ATCC 27562 and CDC 2040-81. *Vibrio parahaemolyticus* 477, a Kanagawa-positive clinical isolate, was obtained from R. M. Twedt, Food and Drug Administration, Cincinnati, Ohio.

Testing of isolates. Isolates were tested with species-specific antiflagellar *V. vulnificus* serum (21) supplied by R. Siebeling, Louisiana State University, Baton Rouge. Anti-H serum was used in two procedures, a tube agglutination test (51°C, 18 h) and a slide agglutination test in which the antiserum had been absorbed onto Cowan I *Staphylococcus aureus* cells. Isolates were grown overnight on tryptic soy agar slants (Difco) at 35°C. Cell suspensions were made by using formalinized (0.3%) 0.15 M phosphate-buffered saline.

TABLE 1. Estuaries of the U.S. West Coast from which *V. vulnificus* was isolated, by sample type

Site (total no. of samples)	No. of positive samples		
	Water	Sediment	Shellfish
California (265)			
Mission Bay		2	
Morro Bay	3		
San Francisco Bay	1	1	
Suisan Bay	1		
Tomales Bay		1	
Arcata Bay	2	1	1[a]
Oregon (160)			
Yaquina Bay	1	4	
Coos Bay	9	2	1[b]
Washington (112)			
Willapa Bay		1	

[a] Mussel (*M. edulis*).
[b] Oyster (*C. gigas*).

A cytotoxin-hemolysin genetic probe for *V. vulnificus* (24) was supplied by J. G. Morris, University of Maryland School of Medicine, Baltimore. The probe was radiolabeled with ^{32}P. *V. vulnificus* isolates were spotted onto plates of tryptic soy agar with 1.5% added NaCl. After overnight incubation at 35°C, colony blots were made using Whatman 541 paper (14). Lysing of colonies and hybridization with cytotoxin-hemolysin probe followed the procedure of Hill et al. (7).

Isolates were tested for mouse lethality by injecting live cultures grown in brain heart infusion (Difco) into treated and untreated 20-g Swiss Webster mice (16). Treated mice were injected intramuscularly with 5 mg of iron dextran (Merrill Dow Pharmaceuticals, Inc., Cincinnati, Ohio) 2 h before injection of the test strains (A. L. Reyes, J. T. Peeler, C. H. Johnson, P. L. Spaulding, and G. N. Stelma, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, P2, p. 275).

Test strains were grown for 8 h at 35°C with shaking in brain heart infusion, centrifuged, and washed three times in 0.15 M phosphate-buffered saline (pH 7.0). Dilutions were prepared in phosphate-buffered saline, CFU were determined in brain heart infusion–2% NaCl pour plates, and 0.5-ml samples of decimal dilutions of cultures were injected intraperitoneally into each of three iron-treated mice. Mice were observed for 72 h. Fifty percent lethal dose values of isolates were calculated for iron-treated mice only, by the procedure of Reed and Muench (18).

RESULTS AND DISCUSSION

V. vulnificus was isolated from 31 of the 529 samples analyzed (5.9%). The incidence by state and samples type is presented in Table 1. This pathogen was isolated from 10 of the 24 distinct estuaries sampled. Most isolations were from Oregon with Coos Bay the most productive, yielding 12 positive samples. Salinity and temperature of water samples which contained *V. vulnificus* ranged from 0.8 to 34‰ and 13.0 to 31.0°C, respectively, and would not explain the higher incidence in Oregon estuaries. Most water with *V. vulnificus* were in the 15 to 30‰ range and >15°C, similar to the conditions reported from other studies (12, 20). *V. vulnificus* was recovered from only two shellfish samples, a mussel in California and an oyster in Oregon. A low incidence in the state of Washington may be explained in part by the generally lower water temperatures found there during the study (11).

Of 44 *V. vulnificus* isolates tested with the species-specific antiflagellar serum (22), 42 agglutinated by either the tube procedure, the slide procedure, or both. Two isolates identified biochemically as *V. vulnificus* failed to agglutinate by either test, as did the reference strain, CDC 2040-81. This clinical strain has also been nonreactive in agglutination tests until manipulated to enhance motility (R. Siebeling, personal communication). Since 95% of the test strains reacted with the antiserum, the use of the agglutination test in a screening procedure or identification scheme would aid in the rapid analysis of samples for this species.

All *V. vulnificus* isolated from environmental samples and all reference strains hybridized with the cytotoxin-hemolysin gene probe, which suggests that all environmental strains produce cytotoxin. Tison and Kelly (23) found that environmental and clinical strains produced cytotoxin; however, titers of cytotoxin produced by environmental strains varied. Our results suggest that the gene probe is a useful screening test for identification of environmental strains but does not indicate the degree of virulence.

TABLE 2. Lethality of *V. vulnificus* isolates from the U.S. West Coast

Strain	Source	Mouse lethality[a]		Cytotoxin probe[b]	Anti-H agglutination[c]	LD_{50} (CFU) for treated mice
		Untreated	Iron treated			
V. vulnificus						
LA M624	Clinical	+	+	+	+	4.9
85A05567	Clinical	+	+	+	+	107
ATCC 27562	Clinical	+	+	+	+	100
CDC 2040-81	Clinical	+	+	+	−	>50,000
C36	Sediment	+	+	+	+	7,000
C153	Water	+	+	+	+	230
C182	Sediment	+	+	+	+	7.6
C265	Sediment	+	+	+	+	66
OR54	Sediment	+	+	+	+	1,600
OR156	Oyster	+	+	+	+	360
WA32	Sediment	+	+	+	+	>20,000
V. parahaemolyticus						
477 (Kanagawa positive)	Clinical	+	+	−	−	>50,000

[a] Mice were injected with approximately 10^8 CFU.
[b] Genetic probe results using cytotoxin-hemolysin gene probe (24).
[c] Agglutination (slide and tube) with species-specific antiflagellar *V. vulnificus* serum (21).

Of 40 isolates injected into untreated mice to test for lethality, 24 were lethal at 10^8 CFU, killing at least two of three mice injected within 72 h. All 40 isolates were lethal (three of three deaths) when injected into iron-treated mice. Fifty percent lethal dose values of seven *V. vulnificus* isolates from different samples ranged from 7.6 to >20,000 CFU in iron-treated mice (Table 2). Four isolates, including the strain isolated from oysters, had low 50% lethal doses, comparable to three of the clinical strains. Comparable lethality between clinical and environmental strains was also reported by Tison and Kelly (23), and our data support that finding.

A relatively low incidence of *V. vulnificus* was found in estuaries of the West Coast compared with the Gulf and Atlantic Coasts. However, the isolation of this organism from mussels and oysters suggests a potential hazard to shellfish consumers since isolates were comparable in lethality to reference clinical strains. Although West Coast shellfish have not been implicated in infection of humans by this pathogen, the presence of virulent strains in these estuaries may be a hazard to users of marine beaches and consumers of contaminated raw or undercooked seafoods, particularly to persons with liver disorders. Antiflaggelar *V. vulnificus* serum and DNA colony hybridization were useful as compared with standard biochemical methods for screening environmental isolates of *V. vulnificus*.

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