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Sandwich Enzyme-Linked Immunosorbent Assay for *Vibrio vulnificus* Hemolysin To Detect *V. vulnificus* in Environmental Specimens

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***Vibrio vulnificus* hemolysin, purified by quantitative isoelectric focusing, was used to prepare rabbit and goat anti-hemolysin. The resulting antibodies were used as capture and detector antibody reagents in a sandwich enzyme-linked immunosorbent assay (ELISA) to detect *V. vulnificus* in environmental samples. By this technique, 4 laboratory-maintained *V. vulnificus* strains and 33 environmental *V. vulnificus* isolates were detected. Also, the technique distinguished five other *Vibrio* species from *V. vulnificus*, and when it was used in combination with colistin-polymyxin-cellobiose agar, 31 non-*V. vulnificus* isolates were excluded. This sandwich ELISA compared favorably with the current Food and Drug Administration standard immunoassay in confirming presumptive *V. vulnificus* colonies from environmental specimens: oysters, sediment, and seawater. Among 340 presumptive *V. vulnificus* colonies, the sandwich ELISA detected 95% of the confirmed *V. vulnificus* colonies. Equally important, the technique correctly distinguished 99% of the non-*V. vulnificus* colonies. The sandwich ELISA offers time-saving and labor-saving advantages over the currently accepted immunoassay.**

The American oyster (*Crassostrea virginica*) industry is threatened because of public health concerns over *Vibrio vulnificus* in raw oysters. This gram-negative bacterium causes severe illness and mortality in susceptible consumers. Representative isolates can be confirmed biochemically as *V. vulnificus* by a standard identification scheme that takes many days (36); however, many phenotypic traits are variable (24, 28, 42). There is much research toward developing species-specific immunoassays to detect *V. vulnificus* in clinical and environmental samples (5, 23, 24, 31, 32, 35).

An antigenic protein, *V. vulnificus* hemolysin (VVH), is produced in maximal amounts at the mid- to late-exponential growth phase (8, 15, 25, 37). VVH is apparently specific to *V. vulnificus*. A radiolabeled probe to the VVH gene provides specific confirmation of both environmental and clinical isolates of *V. vulnificus* (12, 21, 43). Hemolysin production has been reported in all *V. vulnificus* isolates tested by numerous investigators, including 12 isolates tested by Johnson and Calia (11), 44 isolates tested by Kaysner et al. (12), 16 isolates tested by Morris et al. (21), 33 isolates tested by Morris et al. (22), 16 isolates tested by Okada et al. (25), and 40 isolates tested by Tison and Kelly (38).

This research seeks to (i) produce reagent antibodies to VVH, (ii) develop a sandwich enzyme-linked immunosorbent assay (ELISA) for VVH to detect *V. vulnificus* isolates, and (iii) evaluate the sandwich ELISA as a method for detection and enumeration of *V. vulnificus* in oysters and other environmental specimens.

MATERIALS AND METHODS

***V. vulnificus* isolates.** *V. vulnificus* 27562, the type strain, was obtained from the American Type Culture Collection, Rockville, Md.; strain E4215 was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga.; and strain 1001 was obtained from Louisiana State University, Baton Rouge. The Department of Marine Biology, Texas A&M University at Galveston, provided strain 4916 and other environmental presumptive *V. vulnificus* isolates.

Stock cultures were grown on Columbia agar plates overnight at 37°C and collected with 0.85% saline solution. Decimal dilutions were measured for A_{690} and spread plated on heart infusion agar to obtain a standard concentration curve for each of three strains (27562, E4215, and 1001) of the bacteria to estimate subsequent inoculum concentrations of *V. vulnificus.*

Preparation and purification of VVH. The VVH from *V. vulnificus* E4215 was prepared and purified by published methods (8, 14) with the following modifications. Culture medium was the filtrate from heart infusion broth prepared by ultrafiltration in a TCF 10 stirred-cell unit equipped with a YM10 membrane (Amicon, Inc., Beverly, Mass.). The Sephadex G-75 gel filtration step was omitted because of the inefficiency of this procedure observed in preliminary experiments. Also, 0.1 mM EDTA (tetrasodium salt) was included in the buffers to chelate potentially contaminating heavy metals (6) and improve the stability of the VVH.

Protein concentrations were estimated by the Bradford method with bovine gamma globulin as the standard (2). Samples were assayed for microplate hemolysis by using serial twofold sample dilutions in flat-bottom microassay plates and an equal volume of 0.7% sheep erythrocyte suspension in phosphate-buffered saline (PBS; 0.02 M Na_2HPO_4, 0.15 M NaCl [pH 7.4]) containing 0.1% bovine serum albumin (15, 41, 44). The microplate was incubated for 1 h at 37°C (41) and then monitored for A_{690} on a microplate reader (44).

Vertical-slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (16) with a Protean II electrophoresis cell (Bio-Rad, Richmond, Calif.). For reduced VVH analysis, 20 μg of VVH was mixed with sample buffer containing 5% β-mercaptoethanol and boiled for 2 min. For nonreduced VVH analysis, VVH was mixed with sample buffer without reducing agent and without heat denaturation. Following SDS-PAGE, the separated protein bands were transferred to 0.2-μm-pore-size nitrocellulose paper with a Trans-Blot electrophoretic transfer cell (Bio-Rad). Between each step of the Western immunoblot development, the paper was washed three times in washing solution, consisting of 0.01 M Tris and 0.5 M NaCl (pH 7.5) containing 0.1% Tween 20 (TBS-T). Nonspecific binding was blocked for 1 h at room temperature in blocking solution, consisting of TBS-T containing 1% nonfat dry milk (Carnation, Los Angeles, Calif.) and 0.1% normal goat serum. The blot was developed by successive 2-h incubations at room temperature with detector antibody (rabbit whole serum anti-VVH) diluted 1:500 in blocking solution and developer antibody (alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G [IgG] [whole-molecule specific; Bio-Rad]) diluted 1:1,500 in blocking solution. Bound conjugate was observed by incubation for 8 min at room temperature in the substrate 5-bromo-4-chloro-3-indolyl phosphate with nitroblue tetrazolium salt solution (BCIP/NBT substrate kit; Zymed, South San Francisco, Calif.), and the reaction was stopped with water washes.

Preparation of reagent antibodies. Two New Zealand White rabbits were initially immunized subcutaneously at 56°C for 30 min with heat-inactivated VVH at 5 μg per rabbit and an equal volume of Freund's complete adjuvant (FCA). Over the next 18 months, subsequent booster immunizations included Freund's incomplete adjuvant (FIA) with 5 μg of heat-inactivated VVH (four times) and 25 μg of active VVH (twice). One Nubian-mix goat was initially immunized intramuscularly with 50 μg of active VVH with an equal volume of FCA. At monthly intervals, subsequent booster immunizations included FIA with 50 μg of active VVH (three times).

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For monoclonal antibody production, four female BALB/c mice were initially immunized subcutaneously with FCA and heat-inactivated VVH at levels ranging from 1 to 25 μg per mouse. The VVH was inactivated for primary immunization to avoid the lethal effects of active VVH on mice (8, 26, 30). Subsequent booster immunizations included FIA with either heat-inactivated or active VVH and either subcutaneous or intraperitoneal routes of administration. At 3 days prior to hybridoma fusions, animals were given a final intravenous boost of either 1 μg of heat-inactivated VVH or 0.3 μg of active VVH diluted in PBS. Splenocytes from primed mice were fused to myeloma cells (SP2/0-Ag14) (American Type Culture Collection) by a standard fusion protocol with 50% polyethylene glycol (molecular weight, ca. 1,000 Sigma, St. Louis, Mo.) (4). Hybridomas were selected with hypoxanthine-aminopterin-thymidine selection medium (9).

Polyclonal IgG was purified from rabbit and goat sera by a two-step procedure: affinity chromatography with CM Affi-Gel Blue (Bio-Rad) followed by 45% saturated ammonium sulfate precipitation. Ammonium sulfate was removed from the IgG fraction by dialysis against PBS.

Polyclonal sera, purified IgG, and hybridoma supernatants were screened for reactivity with VVH by an antibody capture ELISA. Reagents for the antibody capture ELISA were used at 50 μl per well unless otherwise noted. The VVH was diluted to 5 μg/ml in ELISA coating buffer (0.1 M Na_2CO_3 [pH 9.6]) and added to each well of polyvinyl chloride microplates for overnight coating at room temperature. Between each subsequent ELISA step, microplates were washed three times in TBS-T washing solution. The plates were blocked by adding 200 μl of blocking solution (TBS-T containing 1.0% nonfat dry milk) and incubating for 1 h at 37°C. Serial twofold dilutions of test antibody sample in blocking solution were added to wells and incubated for 90 min at 37°C. Enzyme-conjugated developer antibody, diluted in blocking solution, was incubated for 1 h at 37°C. Goat antibodies were developed with 1:2,500-diluted horseradish peroxidase-conjugated rabbit anti-goat IgG (whole-molecule specific) (Cappel, Cochranville, Pa.). Rabbit antibodies were developed with 1:500-diluted alkaline phosphatase-conjugated goat anti-rabbit IgG (whole-molecule specific) (Bio-Rad). Mouse antibodies were developed with 1:2,000-diluted alkaline phosphatase-conjugated goat anti-mouse IgG (whole-molecule specific; Sigma).

Bound alkaline phosphatase conjugate was observed by adding substrate solution (1 mg of *p*-nitrophenyl phosphate [Zymed] per ml dissolved in 0.1 M 2-amino-2-methyl-1,3-propanediol [pH 10.3]). Following a 20-min incubation at room temperature, the wells were measured for A_{405}.

Bound horseradish peroxidase conjugate was observed by adding 100 μl of substrate solution (3,3′,5,5′-tetramethylbenzidine [TMB Microwell Peroxidase Substrate System; KPI, Gaithersburg, Md.]) to each well. Following an 8-min incubation at room temperature, the reaction was stopped by adding 100 μl of 1.0 M H_3PO_4 to each well, and wells were measured for A_{450}.

Sandwich ELISA for purified VVH. Reagents for the sandwich ELISA for purified VVH were used at 100 μl per well unless otherwise noted. Plates were coated overnight at room temperature with capture antibody (rabbit IgG anti-VVH) diluted to 7.5 μg/ml in ELISA coating buffer. Between each ELISA step, microplates were washed four times in TBS-T washing solution. The plates were blocked with 200 μl of blocking solution (TBS-T containing 5.0% nonfat dry milk) for 2 h at 37°C. Triplicate samples of 0, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 ng of VVH diluted in antibody diluent (TBS-T containing 1.0% nonfat milk and 0.1% normal rabbit serum) were added, and the plate was incubated for 1 h at 37°C. Detector antibody (goat IgG anti-VVH, diluted 1:200 in antibody diluent) was added, and the mixture was incubated for 1 h at 37°C. Developer antibody (horseradish peroxidase-conjugated rabbit anti-goat IgG, diluted 1:2,500 in antibody diluent) was added, and the mixture was incubated for 1 h at 37°C. Bound conjugate was observed by adding TMB substrate solution as stop solution and measuring the wells for A_{450} as described above.

Adsorption to decrease nonspecific ELISA background. Preliminary experiments revealed that some non-*V. vulnificus* species would give ELISA signals approaching those of positive *V. vulnificus* cultures. For reducing the nonspecific background ELISA signal, an acetone powder extract was made from a whole-cell bacterial culture by previously described methods (9). *Pseudomonas aeruginosa* was chosen because of the high sandwich ELISA signal it produced in pure culture and the likelihood that pseudomonads will contaminate environmental samples (7, 17, 18, 33). The acetone powder extract was added to 0.5-ml aliquots of the capture antibody and detector antibody in 10- and 25-mg amounts, respectively. Following a 30-min incubation at 37°C, with agitation at 100 rpm, the supernatants were collected by centrifugation (15,000 × *g* for 10 min at 4°C). The detector antibody supernatant was retreated with another 25 mg of the acetone powder extract in the same manner. The resulting adsorbed capture and detector antibody supernatants were used in subsequent sandwich ELISAs on the environmental specimens and presumptive *V. vulnificus* colonies.

Sandwich ELISA for VVH to detect *V. vulnificus* colonies. *V. vulnificus* 27562, E4125, and 1001 were grown overnight at 37°C on modified colistin-polymyxin B-cellobiose (CPC) agar (18, 35). Sandwich ELISA plates were prepared by coating and blocking as described previously except that the once-adsorbed capture antibody, rabbit IgG anti-VVH, was used at 3.5 μg/ml. Following washing, 100 μl of alkaline peptone water (APW [pH 8.5]) (7) was added to each well. Colonies of the three *V. vulnificus* strains were individually inoculated into three replicate wells in each of five replicate capture antibody-coated ELISA plates. The plates were incubated for 3, 4, 5, 6, and 24 h, respectively. The sandwich ELISA was then completed as described above, except that immediately following the in-plate bacterial growth, plates were washed a total of six times and the detector antibody was the twice-adsorbed goat IgG anti-VVH.

A total of 4 laboratory-maintained *V. vulnificus* strains, 50 presumptive *V. vulnificus* isolates, 7 non-*V. vulnificus Vibrio* species, and 7 unrelated gram-negative bacteria were tested for *V. vulnificus* identity confirmation by both the sandwich ELISA and Food and Drug Administration (FDA) standard immunoassay. The unrelated gram-negative bacteria were selected because they were frequently included in reports on common aquatic flora (7, 17, 18, 33).

Environmental specimens. Three oyster reef sites in Galveston Bay were chosen for specimen collection. The reefs were identified as Sammy's Reef, Deer Island Reef, and Confederate Reef (39). In November 1993, oysters (*C. virginica*), sediment, and seawater were collected from all three sites. The seawater temperature at all sites was 16°C.

Oysters were scrubbed under running tap water and rinsed with sterilized water before being shucked. A 100-g sample of oyster was added to a 100-ml volume of sterilized PBS (0.02 M Na_2HPO_4, 0.15 M NaCl [pH 7.4]) and blended at high speed for 90 s. A 2-ml aliquot of this homogenate was diluted with 8 ml of PBS to make the 10^{-1} dilution. Serial decimal dilutions (1 ml added to 9 ml of PBS) were continued through the 10^{-3} dilution. From each of the 10^{-1} through 10^{-3} dilution tubes, 1-ml aliquots were inoculated into three replicate tubes containing 10 ml of APW for use in the three-tube most-probable-number (MPN) determination procedure. Aliquots (2 ml) of the original homogenate were inoculated into each of three replicate tubes containing 40 ml of APW to serve as the 10^{-0} tubes for the MPN procedure. Excess APW diluent in the 10^{-0} tubes served to dilute organic debris and bactericidal factors of the oyster homogenate (10, 27).

Sediment samples were drained of excess water, and 10 g (wet weight) of each sample was vigorously mixed with 10 ml of PBS. The resultant slurry was serially diluted and inoculated into the APW tubes in the same manner as described for oyster samples. Seawater samples were also serially diluted in PBS and inoculated in 10-ml APW tubes in the three-tube MPN pattern.

Following incubation for 12 to 14 h at 37°C, the APW tubes were examined for turbidity indicative of bacterial growth. One loopful from the top 1 cm of turbid APW tubes was streaked onto modified CPC agar (7). The CPC plates were incubated for 18 to 24 h at 40°C and then examined for the presence of *V. vulnificus*-like growth. Typical *V. vulnificus* growth on CPC agar appeared as flat, yellow colonies (due to the fermentation of the cellobiose) ca. 2 mm in diameter (7). Two presumptive *V. vulnificus* colonies were collected from each CPC agar plate and inoculated into individual precoated and preblocked sandwich ELISA plate wells containing 100 μl of APW. Following a 4-h incubation at 37°C, 25 μl of the culture from each well was transferred individually into a separate ELISA plate for use in an FDA standard immunoassay for comparison purposes. The sandwich ELISA was completed as described above, while the FDA standard immunoassay (7, 35) was modified to enhance the specific ELISA signal. Incubations were done at 37°C instead of the published room temperature. Also, nonspecific binding was reduced by diluting antibodies in blocking solution instead of PBS only.

The MPN estimate of *V. vulnificus* per milliliter of seawater or per gram of oyster or sediment was calculated from the number of turbid tubes later producing confirmed *V. vulnificus* growth on the selective agar. A published MPN determination table was used (1).

Statistical analysis. The standard absorbance curves for inoculum estimation were analyzed by simple linear regression line techniques (19). Sandwich ELISAs were compared with the FDA standard immunoassay by a chi-square test for goodness of fit (29).

RESULTS AND DISCUSSION

Preparation and purification of VVH. Preliminary attempts at purification of VVH by isoelectric focusing had been unsuccessful because of the high lability of the protein when adjacent to the anode and cathode solutions. This problem was overcome by loading the sample manually (instead of with a gradient-forming apparatus), with the sample solution segregated from the electrode solutions (40). The total yield of VVH was ca. 1 mg of purified VVH produced per batch. Highly purified VVH showed good stability when stored at −70°C and was inactivated by treatment at 56°C for 30 min, consistent with previous reports (8, 14).

SDS-PAGE of highly purified VVH under nonreducing conditions revealed only one protein band, with an estimated molecular weight range of 50,000 to 60,000 (data not shown). Western blotting results showed that the highly purified intact protein and its reduced, heat-denatured components were reactive toward the polyclonal serum (Fig. 1). The molecular weight of the VVH could not be estimated by Western blot

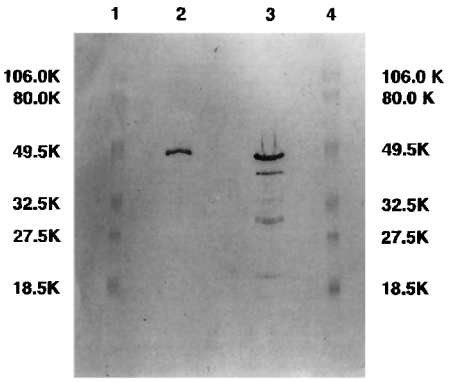

FIG. 1. Western blot of VVH. Lanes: 1 and 4, prestained molecular weight markers (Bio-Rad); 2, VVH (nonreduced); 3, VVH (heat denatured under reducing conditions). Molecular weights (in thousands) are shown at the sides.

because of the relative inaccuracy of the prestained molecular weight markers.

Preparation of polyclonal antibodies. Antibody titers in the rabbits remained low until active VVH was used as the booster immunogen. Following boosts with active VVH, titers reached 1:16,000. Because of the apparent increased immunogenicity of the active VVH, all immunizations in the goat were performed with the native protein. Three total immunizations were required to achieve a titer of 1:4,000. The purified rabbit IgG and goat IgG anti-VVH had equivalent ELISA titers to their original whole-serum counterparts.

Sandwich ELISA for purified VVH. Incubation times for detector and developer steps were standardized at 1 h in consideration of signal quality and time efficiency. The limit of detection was 3.12 ng of VVH, with saturation achieved at 50 ng (Fig. 2).

Sandwich ELISA for VVH to detect *V. vulnificus* colonies. Colony selection from presumptive *V. vulnificus* colonies required more than 3 h for sandwich ELISA detection of VVH production (Table 1). Performing the test cultures in the capture antibody-coated wells led to positive ELISA results even before turbidity development. The growth period of the assay was subsequently standardized at 4 h, but plates could still be processed after overnight incubation if necessary for convenience.

This sandwich ELISA correctly detected a total of 4 laboratory-maintained *V. vulnificus* strains and 33 environmental *V. vulnificus* isolates (Table 2). These isolates were confirmed by

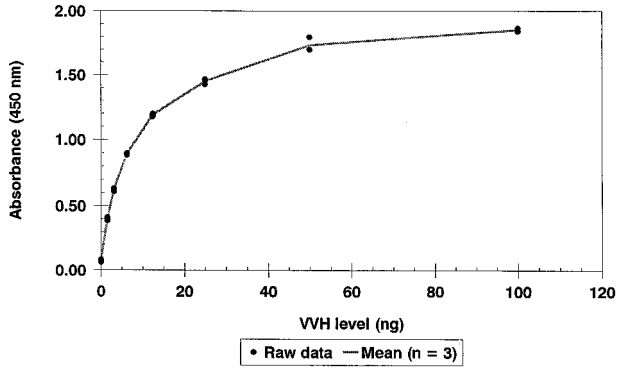

FIG. 2. Limits of detection of VVH by sandwich ELISA. Symbols: ●, raw data; ▨, mean (n = 3).

TABLE 1. Sandwich ELISA confirmation of colony isolates by *V. vulnificus* strain and incubation time

Incubation time (h)	No. of positive wells[a] for *V. vulnificus* strain		
	27562	E4215	1001
3	1	3	3
4	2	3	3
5	3	3	3
6	3	3	3
24	3	3	3

[a] No. of positive wells out of three replicates.

the FDA standard immunoassay. While the adsorption steps with acetone powder extracts were helpful in decreasing background signals, weakly positive signals were still observed for four non-*V. vulnificus* species (*Acinetobacter calcoaceticus*, *Achromobacter icthyodermis*, *Aeromonas hydrophila*, and *P. aeruginosa*) and four unknown environmental isolates. These signaling non-*V. vulnificus* species were easily excluded by their growth pattern on CPC agar (either no growth or cellobiose-negative growth). Other *Vibrio* species (*V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, and *V. cholerae* serogroup O139) and unrelated bacteria were negative on the sandwich ELISA.

Environmental specimens. In conjunction with the CPC selective agar, the sandwich ELISA results compared favorably with the FDA standard immunoassay when used with the environmental specimens (Table 3). A total of 340 presumed *V. vulnificus* colonies were tested, of which 96 were confirmed as *V. vulnificus* growth by the FDA standard immunoassay. The sandwich ELISA correctly detected *V. vulnificus* growth in 91 of these 96 colonies; i.e., it had a 95% sensitivity. The specificity of the sandwich ELISA was 99%, with 242 of 244 colonies correctly distinguished as non-*V. vulnificus*. The positive predictive value of the sandwich ELISA was 98%, with 91 of the 93 positive results being correct. The negative predictive value

TABLE 2. Bacterial species and isolates tested by CPC agar and sandwich ELISA

Bacterial species and isolates	Result[a] in:	
	CPC agar	Sandwich ELISA
Vibrio vulnificus		
Clinical isolates ATCC 27562, E4215, 1001, 4916	+	+
Environmental isolates (33 total)	+	+
Vibrio parahaemolyticus isolate S1	+	−
Vibrio parahaemolyticus ATCC 17802 and isolates S2 and S3	−	−
Vibrio alginolyticus	NG	−
Vibrio anguillarum	NG	−
Vibrio cholerae serogroup O139	−	−
Acinetobacter calcoaceticus	NG	(+)
Achromobacter icthyodermis	NG	(+)
Aeromonas hydrophila	−	(+)
Aeromonas salmonicida	NG	−
Edwardsiella ictaluri	NG	−
Pseudomonas aeruginosa	−	(+)
Escherichia coli	NG	−
Unknown environmental isolates (13 total)	+	−
Unknown environmental isolates (4 total)	−	(+)

[a] Symbols: +, yellow colonies (cellobiose positive); −, Nonyellow colonies (cellobiose negative); NG, no growth on CPC agar; (+), Weakly positive ELISA signal.

TABLE 3. Sandwich ELISA confirmation of CPC cellobiose-positive isolates

FDA immunoassay results	Sandwich ELISA confirmation results		
	No. positive	No. negative	Total no.
Positive	91	5	96
Negative	2	242	244
Total	93	247	340

was also 98%, with 242 of the 247 negative results being correct. Statistical analysis by the chi-square test for goodness of fit showed that the two tests gave similar results ($P < 0.01$). When MPN procedures were applied, the day 3 sandwich ELISA gave identical *V. vulnificus* enumeration results to the FDA standard colony confirmation test on day 4 (Table 4). In addition to the 1-day time savings, the extracellular analyte in the sandwich ELISA technique eliminates the labor of the cell lysis step used in the FDA standard test.

To increase the specificity of the sandwich ELISA, the use of monoclonal antibodies against VVH was attempted. Some of our hybridomas were weakly reactive against VVH in the antibody capture ELISA but were not reactive in the sandwich ELISA. It is possible that in the sandwich technique, the capture antibody blocked necessary VVH epitopes required by the monoclonal antibodies for reactivity. Further attempts at producing better hybridomas may be successful.

This sandwich ELISA technique is very useful at eliminating the lengthy and labor-intensive standard biochemical assays for *V. vulnificus* identification. Evidence of VVH production has been found in all environmental and clinical *V. vulnificus* isolates examined thus far. VVH is not a marker for pathogenicity, since it is produced by both virulent and avirulent strains (11, 12, 22, 37). Arguably, even presumed avirulent *V. vulnificus* could be hazardous to high-risk individuals (3, 34). The extremely high mortality rate associated with *V. vulnificus* septicemia emphasizes the need to prevent infections from occurring (20). Monitoring of oysters should include detection for the family *Vibrionaceae* to reduce the risk of oyster-related infections (13).

TABLE 4. Comparison of *V. vulnificus* enumeration methods for environmental specimens

Specimen description and origin	No. of *V. vulnificus* CFU per g (or per ml)[a] in:	
	Sandwich ELISA isolate confirmation (day 3)	FDA immunoassay isolate confirmation (day 4)
Oysters		
Sammy's Reef	2.3	2.3
Deer Island Reef	0.9	0.9
Confederate Reef	0	0
Sediment		
Sammy's Reef	0.9	0.9
Deer Island Reef	0	0
Confederate Reef	0	0
Seawater		
Sammy's Reef	0	0
Deer Island Reef	0	0
Confederate Reef	0.4	0.4

[a] Estimated by three-tube MPN method.

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