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Direct Plating Procedure for Enumerating *Vibrio vulnificus* in Oysters (*Crassostrea virginica*)

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A procedure for enumerating and identifying *Vibrio vulnificus* in oysters was developed and evaluated. This method consists of growth on a direct plating medium (VVE medium) for isolating the organism from shellfish tissues, followed by biochemical tests for differentiating and identifying presumptively positive isolates. Densities of *V. vulnificus* are reliably obtained in 2 to 4 days, and as few as 10 culturable cells per 100 g can be identified. The procedure was evaluated by using a DNA probe technique specific for the cytotoxin-hemolysin gene of *V. vulnificus* and gas chromatographic analysis of the fatty acid contents of positive isolates. Only 3.2 and 0.4% of the isolates gave false-positive and false-negative results, respectively. The average level of recovery on VVE medium for 33 strains, including both clinical and environmental isolates, was 92% of the level of recovery obtained with brain heart infusion agar supplemented with 1% NaCl. The densities of *V. vulnificus* in oyster homogenates and individual oysters harvested from gulf and Atlantic coastal waters revealed that seasonally high levels occurred. The VVE medium procedure facilitated enumeration of this pathogen in molluscan shellfish and had a distinct advantage over the widely used most-probable-number procedure for *V. vulnificus* enumeration, which requires 5 to 7 days and often gives improbable and imprecise results.

Vibrio vulnificus is a gram-negative, halophilic, oxidase-positive, preferentially fermentative, motile, rod-shaped bacterium that is indigenous to coastal and estuarine waters (6, 7, 17) and has been identified as the etiological agent for certain serious, often fatal, human infections (4, 14). A severe localized infection may result from injuries incurred during the handling of shellfish or after exposure of a preexisting wound to seawater (4). A rapid and frequently fatal primary septicemia may be associated with consumption of raw or improperly cooked oysters (4, 20).

A survey (17) found that *V. vulnificus* is ubiquitous in shellfish, waters, and sediments along the eastern coast of the United States during the summer. Other studies (10, 22) have shown that occasionally high densities of the organism occur in oysters and Gulf Coast waters, especially from May through October. This finding is consistent with the seasonal distribution of clinical cases and fatalities traced to the consumption of raw oysters harvested from these areas (18).

The principal virulence factors of *V. vulnificus* are largely unknown (24), and the relationship between the density of the ingested pathogen and the general health of the human host (with regard to the probability of disease) is unclear. In addition, the numbers of "viable but nonculturable" *V. vulnificus* cells occurring in feral and market oysters, as well as the public health significance of these cells (if any), are not known. The densities of culturable *V. vulnificus* are not indexed by fecal coliforms, the National Shellfish Sanitation Program indicators used to measure the sanitary quality of shellfish and their growing waters (3). Consequently, accurate determinations of the culturable densities of this autochthonous, opportunistic pathogen are essential for developing effective public health safety guidelines.

The selective isolation agar media currently used to identify *V. vulnificus* contain chemical inhibitors and antibiotics to reduce background growth (5, 11–13, 16, 23). These inhibitory compounds significantly decrease the level of recovery of *V. vulnificus*; some target cells fail to grow in their presence. Although those media are useful for isolating and differentiating *V. vulnificus*, their selectivity compromises quantitative recovery and makes them unsuitable for enumerating the organism in environmental samples. The selectivity of these media may also bias results by excluding recovery of certain biotypes, thus possibly leading to inaccurate conclusions concerning the ecology of pathogenic *V. vulnificus*. At the present time, a most-probable-number (MPN) procedure (9, 12) is used to enumerate *V. vulnificus*. Highly selective agar media are used to isolate *V. vulnificus* from presumptive tubes, and differentiation and confirmation of the presumptive isolates require several laborious, time-consuming follow-up measures. Unfortunately, MPN results are generally imprecise compared with the results of direct enumeration procedures. Thus, the MPN method for enumeration of *V. vulnificus* has serious limitations when knowing the densities of this species in shellfish is important. Several other methods for enumerating *V. vulnificus* have been developed recently. An enzyme immunoassay in which monoclonal antibody and culture techniques are used provides a reliable MPN for *V. vulnificus* (21); however, the inherent imprecision of MPNs (25) makes the use of MPN methods less desirable for developing effective safety guidelines. A DNA probe hybridization technique (15) used along with a direct plating procedure is more precise than MPN methods; however, this technique has not been thoroughly evaluated, and its radioactive label prevents its use in many unlicensed shellfish laboratories. A smaller, chromogenic DNA probe which eliminates the need for a radioactive label has been developed (25a), but it too has not been thoroughly evaluated.

In this study we addressed the need to develop and evaluate an accurate, specific, sensitive, simple, direct method to rapidly enumerate and identify *V. vulnificus* in oysters. The

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method described below is intended to recover all *V. vulnificus* strains easily and economically and can be used in virtually all shellfish laboratories.

MATERIALS AND METHODS

Cultures. A total of 28 environmental and 5 clinical isolates of *V. vulnificus* were used to determine levels of recovery. Clinical strains V1000H, EDL174, E4125, and C7184 and environmental strain W108 were provided by J. D. Oliver (University of North Carolina, Charlotte). Clinical strain LAM624 and environmental isolates ALA24 and ALA27 were provided by C. A. Kaysner (Food and Drug Administration, Bothell, Wash.). All other strains were isolated from Gulf Coast and New England oysters (*Crassostrea virginica*). Stock cultures were stored at room temperature in motility medium (10 g of tryptose, 10 g of NaCl, and 5 g of agar in 1 liter of distilled water).

Test suspensions. Test suspensions of the *V. vulnificus* strains used to develop and evaluate the *V. vulnificus* enumeration (VVE) procedure were prepared by inoculating alkaline peptone water (APW) containing 10 g of peptone and 10 g of NaCl in 1 liter of distilled water (final pH 8.5) and incubating the preparations for 18 to 24 h at 35°C. Serial 10-fold dilutions of cultures were prepared in phosphate-buffered saline (PBS) before plating.

Oyster preparation. Eastern oysters (*C. virginica*) from Florida, Alabama, Mississippi, Louisiana, and Texas were received and examined within 24 h of harvest. Oysters obtained from the Cole River in Massachusetts and a limited number of oysters obtained from retail markets along the Gulf Coast were also examined. The oysters were placed in plastic bags and packed in coolers with freezer packs, which maintained the temperatures of the animals between 10 and 15°C until they were received at the laboratory. Meats and liquors were prepared by using recommended procedures (2). Composites of 12 oysters were not diluted before assaying. Meats and liquors from individual oysters were also examined. Single oysters weighing more than 10 g were diluted 1:2 (wt/vol) with PBS, and single oysters weighing less than 10 mg were diluted 1:4 (wt/vol). All oysters were homogenized in a blender at a high speed for 2 min and examined promptly.

Medium preparation. In the VVE procedure a direct spread plate isolation medium, three secondary confirmation media, and two simple biochemical tests are used. VVE medium, the primary spread plate medium, was prepared by adding the following ingredients to 1 liter of distilled water: 10 g of tryptose, 5 g of cellobiose, 20 g of NaCl, 5 g of yeast extract, 0.1 g of lactose, 15 g of agar, 1.0 g of oxgall (Difco Laboratories, Detroit, Mich.), 1 g of K_2PO_4 (added as 4 ml of a 25% aqueous stock solution), 1 g of KH_2PO_4 (added as 4 ml of a 25% aqueous stock solution), and 0.1 g of $MgSO_4 \cdot 7H_2O$ (added as 1 ml of a 10% aqueous stock solution). All stock solutions were filter sterilized through 0.22-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.). After the components were added, the pH of VVE medium was adjusted to 8.5 with 1 N NaOH; the mixture was brought to a boil and autoclaved at 121°C for 15 min. After the mixture cooled to 50°C, the following constituents were added (per liter); 1 g of sodium cholate (Sigma Chemical Co., St. Louis, Mo.), 1 g of sodium taurocholate (Sigma), 0.005 g of potassium tellurite (EM Science, Cherry Hill, N.J.) (added as 1 ml of a 0.5% aqueous solution), 0.1 g of $FeCl_3 \cdot 7H_2O$ (added as 1 ml of a 10% aqueous solution), and 0.1 g of X-Gal (5-bromo-4-chloroindoxyl-β-D-galactopyranoside; Biosynth International, Skokie, Ill.) dissolved in 5 ml of 95% ethyl alcohol and then diluted with 5 ml of sterile distilled water. The entire solution was added aseptically to VVE medium. Solutions of potassium tellurite and ferric chloride were filter sterilized as described above for stock solutions before they were added to the medium. Constituents added to sterile 50°C VVE medium were mixed for several minutes with a magnetic stirrer, and the medium was dispensed into sterile 100- or 150-mm-diameter petri dishes (15 to 20 or 50 to 60 ml per plate). The color of cooled VVE medium is golden tan.

A modified CSPS agar, used as a streak plate medium, was prepared by using the formulation of Kaysner and Tamplin (9) and was further modified by substituting cellobiose for sucrose as the fermentable carbohydrate and omitting the inhibitors sodium dodecyl sulfate and polymyxin B. Modified CSPS agar contained the following ingredients in 1 liter of distilled water: 10 g of proteose peptone, 5 g of beef extract, 15 g of cellobiose, 20 g of NaCl, 0.04 g of bromthymol blue, 0.04 g of cresol red, and 15 g of agar. The pH was adjusted to 7.6. The solution was brought to a boil, autoclaved at 121°C for 15 min, cooled to 50°C, and dispensed into petri dishes. Modified CSPS agar plates are blue to purple.

Ornithine-lysine decarboxylase (OLDC) broth was prepared by adding the following ingredients to 1 liter of distilled water: 10.5 g of decarboxylase base Moeller (Difco), 5.2 g of L-ornithine, 5.2 g of L-lysine, and 10 g of NaCl. The pH was adjusted to 6.0, and the solution was brought to a boil, dispensed into tubes (5 ml of medium per tube), and autoclaved at 121°C for 10 min. Sterile, cooled OLDC broth is typically olive grey.

Nutrient agar was prepared as specified by the manufacturer (Difco) without added salt and was used to differentiate halophilic bacteria from nonhalophilic bacteria.

VVE procedure. In the VVE procedure, the spread plate technique was used with VVE medium for primary isolation, and this was followed by an abbreviated, simple biochemical scheme for differentiating and confirming the identity of a portion of the presumptively positive colonies. VVE medium plates were inoculated with 0.1 to 0.5 ml of oyster homogenate and 10-fold dilutions of oyster homogenate and incubated for 18 to 24 h at 35°C. Typical *V. vulnificus* colonies were raised or flat, circular, and opaque to translucent. Target colonies and other lactose-positive isolates were blue to greenish blue from the hydrolysis of X-Gal, a chromogenic lactose analog present in VVE medium. About 10 or more of the presumptively positive colonies from appropriate VVE medium plates were subcultured on modified CSPS agar. After 18 to 24 h at 35°C, *V. vulnificus* colonies were yellow and either flat or raised. Nonyellow colonies were cellobiose negative. An oxidase test was performed on presumptively positive *V. vulnificus* cultures by transferring a portion of the cellobiose-positive colonies with a sterile hardwood applicator stick, glass rod, or platinum loop onto a filter pad saturated with oxidase reagent (23). Oxidase-positive isolates were identified as presumptive *V. vulnificus* strains, subcultured onto nutrient agar and OLDC broth (overlaid with sterile mineral oil), and incubated at 35°C for 18 to 24 h. The OLDC assay ordinarily was completed within 18 to 24, and a positive reaction (characteristic of all *V. vulnificus* isolates) was light to dark purple. In negative reactions there was either growth but no change from the original color or a bright yellow color. Nonreactive (no color change) tubes were incubated for an additional 24 h. An indole test was performed after the

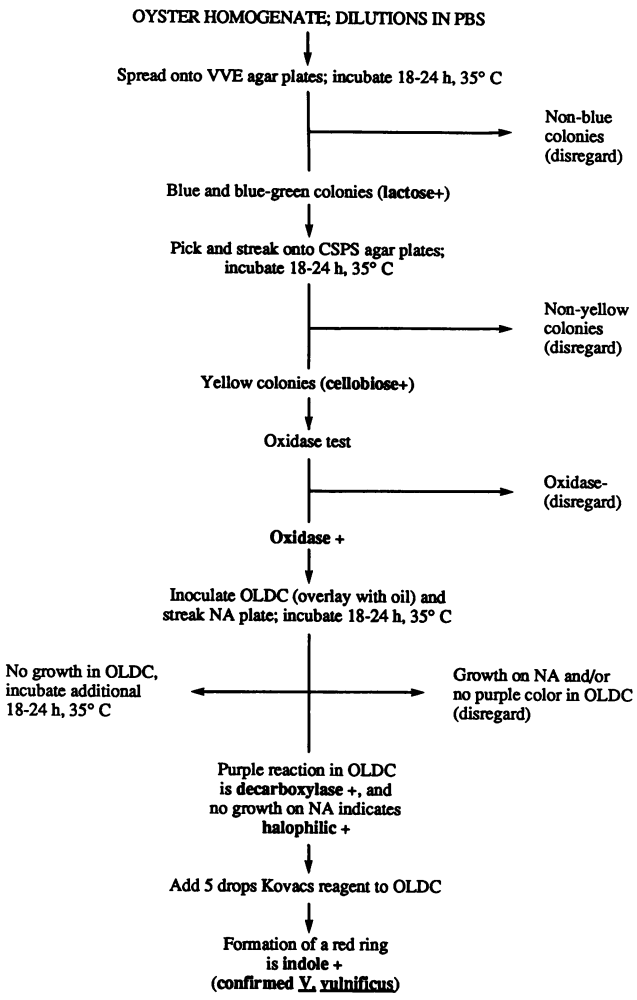

FIG. 1. Flow scheme of the VVE procedure. NA, nutrient agar; OLDC, ornithine-lysine decarboxylase.

OLDC reaction by adding several drops of Kovacs reagent (23) to the same tube. A red ring at the surface was a positive Kovacs test, indicating a confirmed *V. vulnificus* isolate. A flow diagram of the VVE procedure is shown in Fig. 1. The number of *V. vulnificus* colonies on a given VVE medium plate was calculated as follows: (number of biochemically confirmed colonies/number of colonies examined biochemically) × total number of presumptive blue to greenish blue colonies on VVE medium. The densities of this species in oysters were then determined as follows: mean number of *V. vulnificus* colonies on triplicate VVE medium plates × (100/grams of oyster homogenate spread per plate).

Evaluation of VVE procedure. The targeted performance criteria of the VVE method for recovery, specificity, selectivity, and comparability were established before its development, as follows. (i) The lowest acceptable level of recovery was 75% of the level of recovery of *V. vulnificus* cultured on brain heart infusion agar (Difco) supplemented with 1% NaCl. A total of 33 strains of *V. vulnificus* were used to determine levels of recovery. (ii) The specificity limits were established as not more than 10% false-positive and false-negative isolates. False-positive isolates were isolates identified as *V. vulnificus* by the VVE procedure but subsequently found to be members of another species. False-negative isolates were colonies on VVE medium that were not identified as *V. vulnificus* by the VVE procedure (both nonblue background colonies and blue colonies not differentiated as *V. vulnificus*) but were subsequently confirmed to be members of this species. (iii) The targeted selectivity for VVE medium was a 90% reduction in the bacterial growth on marine agar. Individual oysters were examined, and the levels of reduction attributed to VVE medium selectivity were calculated from the differences between the number of CFUs on marine agar and the numbers of CFUs on VVE medium. (iv) The levels of recovery of *V. vulnificus* from feral and market oysters obtained by the VVE procedure were compared with the levels of recovery obtained with the conventional APW MPN procedure, as modified by Kaysner and Tamplin (9).

TABLE 1. Levels of recovery of *V. vulnificus* strains on VVE medium

Source of isolates[a]	No. of strains examined	Mean % of recovery[b]
Clinical[c]	5	102 ± 38
Environmental[d]	10	88 ± 11
Environmental[e]	18	91 ± 16

[a] Clinical strians and environmental isolates initially obtained by the APW MPN procedure were not previously exposed to VVE medium.
[b] The level of recovery for each strain was determined from the mean counts on duplicate plates as follows: (recovery on VVE medium spread plates/recovery on brain heart infusion agar spread plates containing 1% NaCl) × 100. Mean percentages of recovery were calculated by determining the arithmetic average levels of recovery for each group of isolates. Means ± 95% confidence intervals are shown.
[c] Obtained from patients.
[d] Initially isolated on VVE medium.
[e] Initially isolated by the APW MPN method.

Confirmation of the identities of *V. vulnificus* isolates. To determine the specificity of the method, the identities of 221 *V. vulnificus* colonies and 1,982 non-*V. vulnificus* colonies were confirmed by using an isotopic DNA-DNA hybridization probe specific for the cytotoxin-hemolysin gene of *V. vulnificus* (15). Colonies on VVE medium plates were either transferred to modified CSPS agar and directly hybridized with the DNA probe or transferred and grown in motility medium and probed after 20 to 30 colonies were spotted onto tryptic soy agar (Difco) supplemented with 1% NaCl. Radioactive DNA hybridizations (15) were conducted at the Center for Vaccine Development, University of Maryland Medical School, Baltimore.

The identities of some VVE medium isolates whose identities were verified by using the DNA probe were also confirmed by fatty acid analysis, using a Hewlett-Packard gas chromatography system and library (W. L. Landry, Food and Drug Administration, Dallas, Tex.). Species were identified within the genus *Vibrio* and as vibrios and related organisms by targeting specific differences in membrane fatty acid profiles. Isolates obtained from VVE medium or motility medium were spotted onto tryptic soy agar plates until analysis. For fatty acid determinations, isolates were grown on tryptic soy agar at 28°C, saponified, lysed, and methylated. Fatty acid methyl esters were extracted, washed, and determined by using the manufacturer's specifications.

RESULTS

Recovery. The levels of recovery of clinical and environmental *V. vulnificus* isolates on VVE medium are shown in

TABLE 2. Confirmation of the identities of colonies isolated by the VVE procedure

Source of oysters[a]	No. of isolates verified by:			No. of false reactions
	VVE procedure	DNA probe analysis	Fatty acid analysis	
Positive isolates[b]				
Apalachicola, Fla.	1	0	0	1
Dauphin Island, Ala.	38	35	35	3
Ocean Springs, Miss.	58	58	58	0
Davis Bay, Miss.	34	32	32	2
Bay Adams, La.	19	18	18	1
Cole River, Mass.	3	3	3	0
Negative isolates[c]				
Apalachicola, Fla.	6	6	6	0
Dauphin Island, Ala.	24	24	24	0
Ocean Springs, Miss.	9	9	9	0
Davis Bay, Miss.	24	24	24	0
Bay Adams, La.	18	18	18	0
Cole River, Mass.	3	3	3	0

[a] Eastern oysters (*C. virginica*).
[b] Verified as *V. vulnificus* isolates.
[c] Verified as non-*V. vulnificus* isolates.

TABLE 3. Levels of recovery of *V. vulnificus* from oysters by the VVE procedure and the APW MPN method

Oyster no.[a]	No. of cells/100 g		Tube code[c]
	VVE procedure	APW MPN method[b]	
1	<330	<40	000000
2	<330	<40	000000
3	2×10^6	80	110000
4	2×10^6	80	110000
5	2×10^6	120	100200[d]
6	<330	<40	000000
7	1×10^5	108	000120[d]
8	1×10^6	72	000200[d]
9	1×10^6	40	100000
10	4×10^5	108	000021[d]
11	2×10^6	160	100021[d]
12	7×10^5	74	020000

[a] Eastern oysters (*C. virginica*) were obtained from Davis Bay, Miss.
[b] The APW MPN method (9) was performed by using a five-tube, six-dilution series. The MPNs were determined from tables (2).
[c] The tube code indicates the numbers of tubes within a five-tube six-dilution series (10.0, 1.0, 0.1, 0.01, 0.001, and 0.0001 g) from which *V. vulnificus* was isolated.
[d] Improbable tube code (25).

Table 1. A total of 33 *V. vulnificus* strains were examined, and the level of recovery on VVE medium was 92% of the level obtained with brain heart infusion agar supplemented with 1% NaCl, exceeding the stated minimum performance criterion of 75%. The average levels of recovery for environmental strains with and without prior exposure to VVE medium did not differ significantly.

Specificity. Although several colonial morphologies were produced by environmental *V. vulnificus* strains isolated on VVE medium plates, morphology alone was not sufficient for *V. vulnificus* identification. Consequently, the identities of a number of blue colonies had to be confirmed by biochemical tests. Oyster samples to determine the specificity of the VVE procedure were obtained at various Gulf Coast locations during the period from March through December. The results of DNA probe verification studies for both positive and negative colonies isolated by the VVE procedure exceeded our prerequisite minimum performance criteria (data not shown). Of the 221 blue and blue-green isolates initially tested and determined to be positive by the VVE procedure, the DNA probe verification method identified 214 (96%) as *V. vulnificus*, a false-positive rate of 3.2%. Of the 1,982 isolates determined to be negative by the VVE procedure, only 8 were identified by the DNA probe verification method as *V. vulnificus*, a false-negative rate of <1%. These few false results indicated that virtually all *V. vulnificus* colonies on VVE medium are blue or blue-green and that the abbreviated biochemical scheme devised for confirming the identities of these colonies from VVE medium plates is highly reliable for differentiating *V. vulnificus* from other species. An additional set of presumptively positive (blue and blue-green) colonies were picked and tested biochemically and by both DNA probe and fatty acid analysis methods. A total of 153 colonies were identified as *V. vulnificus* colonies and 84 were identified as non-*V. vulnificus* colonies by the VVE biochemical scheme (Table 2). The identities of 146 (95.4%) of the 153 isolates identified as *V. vulnificus* strains were verified by both the DNA probe assay and the fatty acid profile analysis; there were only 7 (4.6%) false-positive isolates. The results obtained for all 84 isolates found to be negative by the VVE procedure were verified by both confirmation techniques. The results of the DNA probe and fatty acid assays were in total agreement for all of the isolates examined.

Selectivity. VVE medium did not meet the goal of reducing background growth by at least 90%. Nine homogenates from oyster samples obtained from April through October were examined, and bacterial colony counts on VVE medium and marine agar spread plates were compared (data not shown). VVE medium selectivity reduced bacterial growth on marine agar by 61 to 99% (mean, 82%). Although less than desired, the modest selectivity of VVE medium permitted isolation of *V. vulnificus* from oysters at densities as low as 1 cell per g on a single large petri plate (150 by 15 mm). During development of VVE medium, many potential selective agents were tested for their ability to reduce background growth; however, because most of the antibiotics, detergents, dyes, and chemical inhibitors tested adversely affected the recovery of *V. vulnificus*, they were not included, and only oxgall, sodium cholate, sodium taurocholate, and potassium tellurite were retained at the concentrations specified above. The modest selectivity of VVE medium means that several biochemical tests must be used for reliable identification of *V. vulnificus*. As shown by the high level of specificity, the tests included in the procedure effectively distinguished the target organisms from other lactose-positive colonies found on VVE medium plates.

Comparability. We consistently recovered significantly higher numbers of *V. vulnificus* from the 12 individual oysters examined with the VVE procedure than with the APW MPN method (9) (Table 3). In addition, an excessive number of improbable tube codes (5 of 12) was found. According to the results of a study of the validity of MPN estimates (25), this number surpassed the maximum permissible number (one) of improbable tube codes for 12 specimens. The APW MPN method, therefore, is considered unreliable for accurate enumeration of this species.

Sensitivity. VVE sensitivity depends on the density of *V. vulnificus* in the specimen and the amount of homogenate examined. For 0.1 g, the lower level of detection by the VVE procedure is 1,000 cells per 100 g (equivalent to 10 CFU of *V.*

TABLE 4. Densities of *V. vulnificus* determined for individual Gulf Coast oysters (*C. virginica*)

Source of oysters	Oyster no.	No. of *V. vulnificus* cells/100 g
Dauphin Island, Ala.[a]	1	2.0×10^4
	2	1.5×10^5
	3	6.0×10^4
	4	6.0×10^3
	5	3.0×10^4
	6	8.0×10^4
St. Joe Reef, Miss.[a]	1	1.6×10^6
	2	1.4×10^5
	3	1.1×10^6
	4	6.0×10^5
	5	1.0×10^6
	6	6.0×10^6
	7	5.0×10^5
	8	9.0×10^5
	9	1.2×10^5
	10	9.0×10^5
	11	4.0×10^5
	12	4.0×10^5
Davis Bay, Miss.[a]	1	8.0×10^5
	2	1.3×10^6
	3	2.4×10^6
Bay Adams, La.[a]	1	1.7×10^6
	2	9.0×10^5
	3	1.8×10^6
	4	2.3×10^6
	5	1.5×10^6
So. Point Bay, La.[a]	1	2.0×10^6
Seafood market, Carabelle, Fla.[b]	1	1.0×10^5
	2	6.0×10^4
	3	4.0×10^5
	4	4.0×10^5
	5	4.0×10^5
	6	5.0×10^5
Seafood restaurant, Apalachicola, Fla.[b]	1	2.0×10^4
	2	1.0×10^3
	3	1.0×10^5
	4	1.0×10^3
	5	2.0×10^4
	6	2.0×10^4

[a] Freshly harvested oysters.
[b] Retail oysters purchased chilled.

vulnificus per g). Increasing the amount of oyster homogenate examined enhances the sensitivity of the method. A maximum of 0.5 g of shellfish homogenate can be examined on a standard petri plate (100 by 15 mm), providing a level of detection of 200 cells per 100 g. When very low densities of the species are anticipated, a larger petri plate (150 by 15 mm) can be used for up to 2 g of homogenate, thereby increasing the level of detection to 50 cells per 100 g. Using several large plates enhances the level of detection proportionately; e.g., if a 10 g of homogenate is examined on five large plates, the level of detection is 10 CFU of *V. vulnificus* per 100 g.

***V. vulnificus* densities in oysters.** In a field test of the VVE method, the densities of *V. vulnificus* were determined in oysters that were freshly harvested from Gulf Coast waters and oysters that were obtained from retail markets (Table 4). During the summer, more than 10^5 *V. vulnificus* cells per 100 g was often recovered from freshly harvested oysters. Although oysters obtained from seafood markets were refrigerated, they contained 10^3 to 10^5 *V. vulnificus* cells per 100 g. These data demonstrate that from late spring (April) through early fall (October) high densities of *V. vulnificus* are found in both market oysters and oysters freshly harvested from Gulf Coast waters.

DISCUSSION

The VVE procedure is an accurate, specific, and simple method for quantitative enumeration of *V. vulnificus* in oysters. It is superior to the APW MPN procedure with regard to recovery and precision, requiring 2 to 4 days for test completion compared with 5 to 7 days for the MPN technique. The overall level of recovery by the primary isolation medium (92%) well exceeded the preestablished minimum acceptable recovery criterion. The minimum acceptable limits of specificity (not more than 10% false-positive and false-negative results) also were exceeded. Although only moderately selective, a single large VVE medium spread plate permitted isolation of 50 CFU of *V. vulnificus* per 100 g of oyster meat. This level of detection was more than adequate for monitoring *V. vulnificus* densities in oysters from the Gulf Coast region, at least from spring through fall, when concern for *V. vulnificus* in oysters from this region is greatest.

The DNA probe (15) for the cytotoxin-hemolysin gene (26) of *V. vulnificus* was extremely valuable for evaluating the specificity of the VVE procedure. With the probe technique, *V. vulnificus* can be enumerated directly from VVE medium or from any medium on which this organism can be recovered. The major drawback of the probe is the use of the radioactive label ^{32}P, which requires specific facilities and training for routine use. Most laboratories that monitor shellfish quality do not have this capability. A recently developed, smaller, chromogenic probe (25a) should solve this problem, but this probe has not been thoroughly investigated and assay costs may be substantial. Use of the chromogenic probe with VVE medium should eliminate the need for subsequent biochemical assays for isolates and permit reliable enumeration of *V. vulnificus* in oysters within 24 h.

Colony opacity on VVE medium varied among isolates belonging to several isogenic strains of *V. vulnificus*. Two distinct morphological types were recognized, an opaque variant characterized by a thick mucopolysaccharide capsule surrounding the cell and a translucent variant which lacked a visible capsule (1, 27). In investigations of lethal dose values for mice (19), the opaque encapsulated strains were much more virulent than the nonencapsulated strains. The ability of the VVE procedure to recover, enumerate, and distinguish the pathogenic opaque strians is highly desirable.

The densities of *V. vulnificus* determined for Gulf Coast oysters (both freshly harvested oysters and oysters obtained from seafood markets) indicate that millions of cells of this opportunistic pathogen are ingested frequently by individual consumers of raw oysters. Nevertheless, the levels of occurrence of illness and death attributed to this organism are relatively low. We speculate, therefore, that a lethal dose, even for health-compromised individuals (18, 20), may be quite high. The high numbers of *V. vulnificus* that occur naturally in oysters may represent an enhanced threat to compromised individuals when improper handling conditions allow the organism to multiply within the shellfish. In a recent study (8). *V. vulnificus* numbers increased when oysters were stored at temperatures above 10°C. Thus, the densities of this species in oysters must be accurately

monitored throughout the commercial handling process. This, in turn, requires the determination of time and temperature parameters which enable viable but nonculturable cells to revert to an infectious, culturable state.

Determining the relationship between the numbers of *V. vulnificus* present in marine foods and the resulting human health risk is essential for developing effective safety measures. Ultimately, public health officials must determine how many of these pathogens (if any) can be safely consumed. The VVE procedure is a reliable, simple, inexpensive method for enumerating *V. vulnificus* in oysters and is readily availble to any bacteriology laboratory concerned with ecological or epidemiological studies of this organism.

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