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Rapid Identification of *Vibrio vulnificus* on Nonselective Media with an Alkaline Phosphatase-Labeled Oligonucleotide Probe

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An oligonucleotide DNA probe (VVAP) was constructed from a portion of the *Vibrio vulnificus* cytolysin gene (*hlyA*) sequence and labeled with alkaline phosphatase covalently linked to the DNA. Control and environmental isolates probed with VVAP showed an exact correlation with results obtained with a plasmid DNA probe (derived from pCVD702) previously described as having 100% specificity and sensitivity for this organism. Identification of *V. vulnificus* strains was confirmed independently by analysis of the cellular fatty acid composition and by API 20E. Naturally occurring *V. vulnificus* bacteria were detected without enrichment or selective media by VVAP in unseeded oyster homogenates and seawater collected from a single site in Chesapeake Bay during June at concentrations of 6×10^2 and 2×10^1 bacteria per ml, respectively. *V. vulnificus* bacteria were also enumerated by VVAP in oysters seeded with known concentrations of bacteria and plated on nonselective medium. The VVAP method provides a rapid, accurate means of identifying and enumerating *V. vulnificus* in seawater and oysters without the use of selective media or additional biochemical tests.

Vibrio vulnificus is a human pathogen that is common to the estuarine environment (4, 6, 14, 15, 21, 22). It has been identified as the causative agent of a potentially fatal septicemia (mortality, >50%) which occurs following the ingestion of raw oysters, and sometimes severe but usually nonfatal wound infections have resulted from the handling of shellfish or from exposure to seawater (1, 7, 11, 16, 20, 22–24). Although infection can occur in healthy individuals, disease is usually associated with underlying conditions such as cirrhosis, diabetes, and hemochromatosis.

Previously, we reported the use of a 3.2-kb ^{32}P-labeled DNA fragment containing the *V. vulnificus* cytolysin gene as a genetic probe (12, 13, 25). This probe showed 100% specificity and sensitivity for clinical isolates of *V. vulnificus* as well as environmental isolates that had been identified as *V. vulnificus* by DNA-DNA hybridization (12), results which were confirmed by Kaysner et al. (6). We subsequently sequenced the hemolysin-cytolysin structural gene, *vvhA* (27). In the present study, we evaluated a method for the detection of *V. vulnificus* in samples from the environment that involved the use of an alkaline phosphatase-labeled oligonucleotide probe derived from a DNA sequence within the *vvhA* gene. This method permits rapid isolation and enumeration of the organism without enrichment or selective media.

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MATERIALS AND METHODS

Bacterial strains and media. Control strains from our culture collection included the following *Vibrio* species (with numbers of strains in parentheses): *V. vulnificus* (18), *V. fluvialis* (6), *V. parahaemolyticus* (8), *V. mimicus* (4), *V. hollisae* (3), *V. harveyi* (1), *V. cholerae* O1 (4), non-O1 *V. cholerae* (16), *V. furnissii* (1), *V. metschnikovii* (1), *V. damsela* (2), and *V. alginolyticus* (1).

V. vulnificus M06-24 and LAM624 were used as positive controls, and *V. fluvialis* 807-77 and *V. cholerae* NRT36S or 569B were negative controls in this study. Strains were stored at −70°C either in L broth with 50% glycerol (1.5 ml) or in microtiter wells in L broth (200 μl) with 10% dimethyl sulfoxide. Reagents for media were purchased from Difco (Detroit, Mich.); chemicals, unless otherwise noted, were obtained from Sigma (St. Louis, Mo.).

Filter preparation. Colony blots were prepared from bacteria grown on agar for 16 to 72 h at room temperature or 30°C. Colonies were transferred to filter paper (no. 541; Whatman, Inc., Clifton, N.J.) by overlaying agar plates for 1 h. Filters were microwaved (500 W, 1 to 3 min) in alkaline buffer (0.5 M NaOH–1.5 M NaCl) as previously described (3). Our procedure also included additional treatment in neutralization buffer (2 M ammonium acetate) at room temperature for 5 min followed by two washes in 0.15 M NaCl–0.015 M sodium citrate (SSC), pH 7.0. Additionally, some filters were processed with proteinase K treatment (40 μg/ml of SSC at 42°C for 30 min) to remove background alkaline phosphatase activity and, subsequently, washed twice for 10 min each in SSC at room temperature and air dried. Filters have been stored for up to 6 months before hybridization.

pCVD702 gene probe. The 3.2-kb *Hin*dIII-*Eco*RI fragment of pCVD702 (24) containing the *V. vulnificus* cytolysin gene

was isolated from agarose gel and labeled with ^{32}P by the random primer method according to the manufacturer's (Bethesda Research Laboratories, Gaithersburg, Md.) specifications. Colony blots probed with the pCVD702 probe were prepared on no. 541 filters that were microwaved as described above or on nitrocellulose as described previously (12). All strains were probed in triplicate with colony blots of appropriate control strains developed simultaneously.

Alkaline phosphatase-labeled probe. The *V. vulnificus* alkaline phosphatase-labeled gene probe (VVAP) was derived from the sequence of the *V. vulnificus* cytolysin structural gene, *vvhA*, and consisted of the following oligonucleotide sequence (nucleotides 1857 to 1880 [from the sequence published in reference 27]): GAGCTGTCACGGCAGTTGG AACCA. Alkaline phosphatase-labeled probe was either purchased from Molecular Biosystems, Inc., San Diego, Calif., or constructed by using E-Link (Cambridge Research Biochemicals, Wilmington, Del.) to attach the alkaline phosphatase to a 5′ amino oligonucleotide synthesized at the Biopolymer Laboratory, University of Maryland at Baltimore.

Hybridization buffer consisted of SSC with bovine serum albumin (0.5%), sodium lauryl sulfate (1%), and polyvinylpyrrolidone (0.5%). Filters were preincubated in hybridization buffer (5 to 10 ml per filter) at 50 or 56°C for 30 min; VVAP (10 nM) was added to filters in fresh buffer (prewarmed to hybridization temperature) and hybridized for 30 min or 1 h at 50 or 56°C. Filters were washed twice at 50 or 56°C in SSC with 1% sodium dodecyl sulfate and three times in SSC. Alkaline phosphatase activity was assayed with nitroblue tetrazolium (75 mg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (50 mg/ml) in Tris buffer received from Molecular Biosystems, Inc., or in prewarmed (37°C) diethanolamine (DEA) buffer (100 mM [pH 9.5], with 2 M $MgCl_2$ and 2% NaN_3). Control strains of *Vibrio* species were recovered on L agar from frozen stocks and probed with pCVD702 and VVAP. All strains were probed in triplicate on separate filters with VVAP, and colony blots of control strains were developed simultaneously.

Fatty acid analysis of *V. vulnificus* strains. Computer analysis of gas chromatography of the fatty acid methyl esters of test strains was performed by using a methylphenyl-silicone fused capillary column (25 m by 0.2 mm) (Hewlett-Packard Co., Avondale, Pa.) on a Hewlett-Packard 5898A equipped with a flame ionization detector coupled with a 3392A integrator, a 7673A automatic sampler, and a model 300 computer. Identification of strains was based on both the Hewlett-Packard microbial identification system described by the manufacturer and a new library that had been constructed from the profiles of 291 environmental and 13 clinical isolates of *V. vulnificus* (8).

Identification of *V. vulnificus* isolated from the environment. Bacteria were obtained from seawater samples and oyster homogenates. Oyster homogenates were prepared by blending together 3 to 12 oysters, which were diluted 1:1 in sterile Dulbecco's phosphate-buffered saline (PBS) in a Waring blender for 90 s; results are reported as numbers of bacteria per milliliter of undiluted oyster homogenate (ca. 1 g of oyster meat per ml). Samples were collected in the summer from either Apalachicola Bay in Florida or from Chesapeake Bay in Maryland.

Samples of water and oyster homogenates were serially diluted in PBS, and 200 μl was spread on *V. vulnificus* enrichment agar (VVE) (9), *V. vulnificus* agar (VVA) (10), or Luria agar (L agar). VVE is L agar supplemented with 5 g of NaCl, 1 g of cellobiose, 0.1 g of lactose, 1 g of oxgall, 1 g of K_2HPO_4, 1 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 1 g of Na cholate, 1 g of Na taurocholate, 0.005 g of potassium tellurite, 0.1 g of $FeCl_3 \cdot H_2O$, and 0.1 g of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (pH 8.5) per liter. On VVE, possible *V. vulnificus* (lactose-positive) organisms were detected as blue colonies, indicating the hydrolysis of a β-galactosidase. VVA contains 30 g of NaCl, 10 g of cellobiose, 20 g of proteose peptone, and 0.6 g of bromthymol blue (pH 8.0) per liter, with 25 g of agar per liter; possible *V. vulnificus* organisms were identified as yellow, cellobiose-fermenting colonies (nonfermenters form green colonies). Plates were incubated at 35°C overnight or at room temperature for 24 to 72 h, and possible *V. vulnificus* colonies were picked and transferred to L agar for hybridization with probes and for analysis of fatty acid composition. Selected cellobiose-positive and -negative colonies from VVA were further identified biochemically by using API 20E (Analytab Products, Plainview, N.Y.). Colonies were picked from the L agar spread plates to L agar grids for hybridization with VVAP. The latter colonies were also replica plated to thiosulfate citrate bile salt sucrose agar (TCBS; Difco) and observed for growth and sucrose fermentation on this medium.

Direct enumeration of *V. vulnificus* in oysters and seawater. Water and oyster samples were collected in June 1991 and March 1992 from the same site in Chesapeake Bay. Oyster homogenates (prepared as described above) and seawater were serially diluted 1:10 in PBS, and undiluted or diluted samples (200 ml) were plated immediately after collection on either L agar or VVA. Plates were incubated at room temperature, and the total numbers of viable heterotrophic bacteria were determined from colony counts on agar after 72 h of incubation. Plates containing between 20 and 1,000 colonies were overlaid with no. 541 filters and hybridized with the VVAP probe, and the number of probe-positive isolates was determined as described above.

To assess the accuracy of VVAP as a detection method, undiluted samples of seawater or oyster homogenates (prepared as described above except without dilution in PBS) were seeded with serial 1:10 dilutions of *V. vulnificus* M06-24 in PBS. The concentration of the inoculum was determined by plating the serial dilutions of pure cultures of *V. vulnificus* in duplicate on L agar. Individual aliquots of seawater or homogenates were seeded with different concentrations of *V. vulnificus*, ranging from 0 to 1.4×10^4 bacteria per ml. The total number of bacteria was determined individually for each of the seeded samples by plate counts on agar. Seeded samples were serially diluted 1:10 in PBS, and 100-μl dilutions were plated in duplicate on L agar; colonies were counted after incubation at room temperature for 72 h. VVAP-positive bacteria were enumerated directly by colony blots of the L agar plates used to determine total bacteria, and blots were developed as described above. Colony blots of the inoculum were also developed as a positive control.

Nucleotide sequence accession number. The nucleotide sequence for gene *vvhA* is available under GenBank accession no. M34670.

RESULTS

Optimization of the VVAP method. Optimal results were obtained when *V. vulnificus* DNA was hybridized with the VVAP probe at 56°C for 1 h. Cross-reactivity with other vibrios, particularly some strains of *V. fluvialis*, was observed with lower hybridization temperatures (50°C); however, *V. vulnificus* always gave a visibly stronger signal,

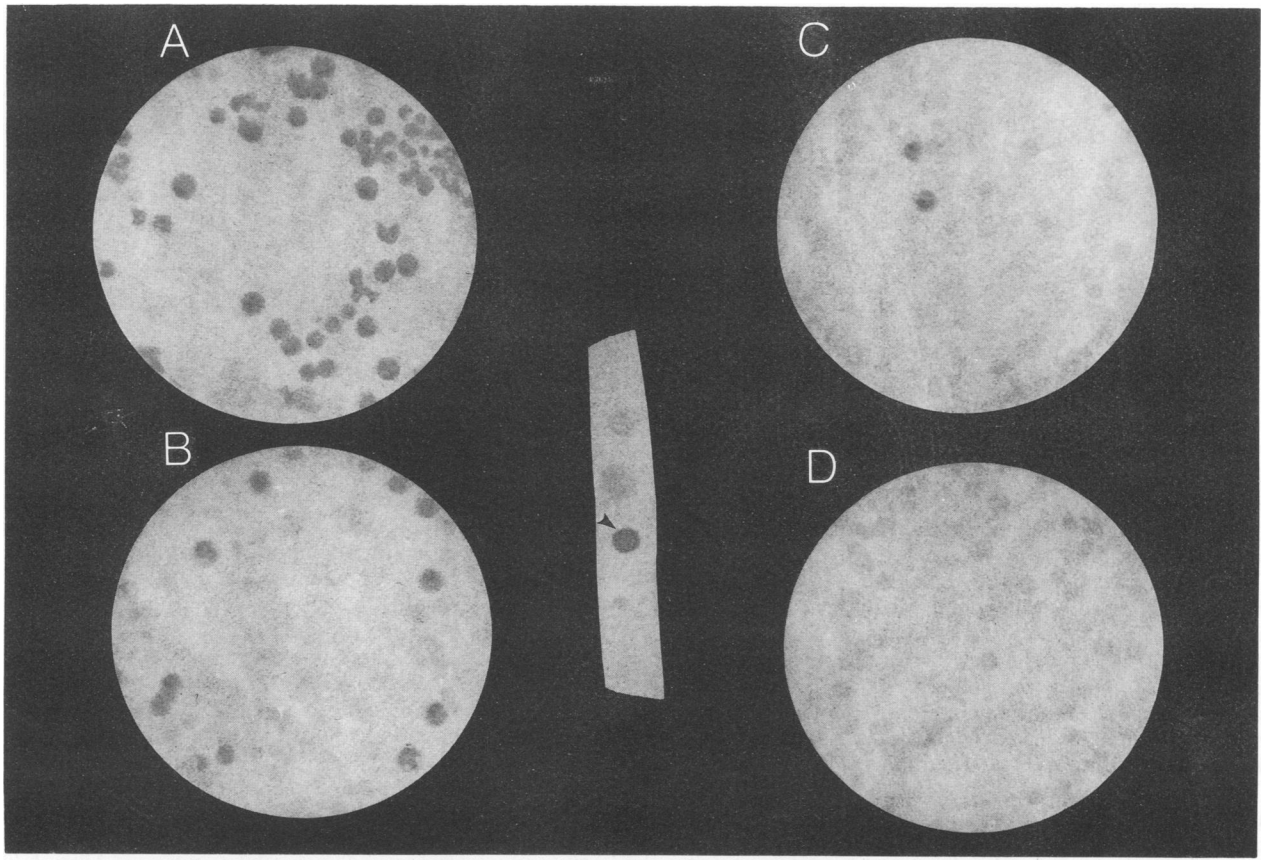

FIG. 1. VVAP method of colony blot hybridization of L agar plates inoculated with 100 μl of serial 10-fold dilutions of *V. vulnificus* in undiluted oyster homogenates. Expected counts (based on inocula) (with actual counts in parentheses): (A) 140 (65), (B) 14 (13), (C) 1 (2), (D) 0 (0). Center strip, a colony blot of control strains (from top to bottom): *V. cholerae* NRT36S, *V. fluvialis* 807-77, *V. vulnificus* M06-24 (arrowhead), and *V. cholerae* 569B.

even at 50°C, and cross-reactivity was eliminated at 56°C. Hybridizations without protease pretreatment resulted in greater background activity; hybridization for a shorter period (30 min) resulted in a decreased signal. Color development in DEA buffer was usually complete in 30 to 60 min. Development in Tris buffer as recommended by Molecular Biosystems, Inc., required at least 4 h.

Colony blots of control strains were developed simultaneously with samples assayed as shown in the center strip in Fig. 1; the darker colony is *V. vulnificus* M06-24. Although not seen in black-and-white photography, negative colonies, which appear light grey in Fig. 1, were actually yellow, and positive colonies, which appear dark grey, were brown to deep purple. Contrast could be enhanced by increasing the amount of probe used for hybridization.

Identification of *V. vulnificus* isolates. VVAP correctly differentiated previously identified *V. vulnificus* from other *Vibrio* species. All 18 of the *V. vulnificus* isolates were positive for VVAP; however, none of the 47 strains from the other 10 *Vibrio* species hybridized with the probe under stringent conditions.

In order to determine whether the VVAP probe could be used to identify a diverse selection of environmental *V. vulnificus* isolates, a total of 110 colonies that were β-galactosidase positive on VVE medium or cellobiose positive on VVA were evaluated by hybridization to both the VVAP and the PCVD702 probes and by fatty acid analysis. Of colonies that were β-galactosidase positive on VVE medium, 66% (52 of 79) were VVAP probe positive. Also, 54% (23 of 41) of the cellobiose-positive colonies isolated on VVA hybridized with the probe. Results obtained with the VVAP method showed 100% agreement with identification based both on the pCVD702 radioactively labeled probe and on analysis of the fatty acid composition of these isolates.

All cellobiose-positive strains that were VVAP positive (n = 23) were further confirmed as *V. vulnificus* by the API 20E system. None of the 16 cellobiose-positive, VVAP-negative strains which survived subculture had an API 20E profile consistent with *V. vulnificus*. Thirty-five cellobiose-negative (green) colonies were streaked for isolation from VVA; none hybridized with the VVAP probe.

Colonies (n = 350) from environmental samples spread on L agar plates were inoculated both to TCBS and gridded L agar plates. The gridded L agar plates were probed with VVAP. Sixty-four of 65 VVAP-positive colonies grew on TCBS. Six (9%) of these 64 strains were sucrose positive. All VVAP-positive strains recovered in this experiment had an opaque colony morphology (a marker for encapsulation and virulence [19, 26]) prior to further subculture.

Direct enumeration of *V. vulnificus* from oyster homogenates and seawater samples plated on nonselective media. The above studies utilized colonies picked from selective and nonselective media. However, the process of picking single colonies for subsequent identification is extremely time-

TABLE 1. Enumeration of *V. vulnificus* and total viable heterotrophic bacteria in seawater and oyster samples collected at a single site in Chesapeake Bay[a]

Sample	Mo	Bacteria/ml			
		Heterotrophic		*V. vulnificus*[b]	
		LA	VVA	LA	VVA
Seawater	June	2.4×10^2	6.3×10^1	2.0×10^1	3.0×10^1
	March	6.2×10^2	ND	0	ND
Oysters[c]	June	7.2×10^4	5.4×10^3	6.2×10^2	6.7×10^2
	March	8.5×10^3	ND	0	ND

[a] Abbreviations: LA, L agar; ND, not done.
[b] Determined by colonies that were positive for the VVAP probe.
[c] Results are expressed as numbers of bacteria per milliliter of undiluted oyster homogenate; 1 ml of undiluted oyster homogenate is equal to approximately 1 g of oyster meat.

consuming. In an effort to avoid this step and to permit direct enumeration of *V. vulnificus* in test samples, we used the VVAP probe to screen filters prepared from spread plates on nonselective media.

Colony blots were prepared from L agar spread plates of seawater and oyster homogenates without subculture or enrichment. Although the total heterotrophic bacterial population did not show much variation from June to March, the number of *V. vulnificus*, as enumerated by VVAP on L agar, decreased from 6.2×10^2/ml of oyster homogenate and 2.0×10^1/ml of seawater in June to undetectable levels in March (Table 1). Enumeration of *V. vulnificus* by the VVAP probe on VVA was comparable to that on L agar; however, the total bacterial count was reduced about 10-fold on VVA medium. By using the VVAP probe, it was possible to enumerate *V. vulnificus* colonies isolated from oysters in less than 24 h.

To further confirm the accuracy of VVAP enumeration of *V. vulnificus*, undiluted oyster homogenates and seawater were seeded with different concentrations of this organism (Table 2). As noted above, samples obtained in March contained no background levels of *V. vulnificus*, as determined by screening with the VVAP probe. As expected, the total counts of background bacteria in environmental samples remained relatively constant, since aliquots of the same samples of either seawater or oyster homogenate were used for seeding, and except for the highest inoculation, the concentrations of *V. vulnificus* were at levels below background bacterial colony counts (i.e., less than ca. 2×10^3). However, when the inoculum of *V. vulnificus* exceeded 10^4, a 10-fold increase in the total colony count was observed. Colony blots from serial dilutions of *V. vulnificus* in seeded oyster homogenates are shown in Fig. 1. Total numbers of heterotrophic bacteria were determined from colony counts on agar and not from blots. Spread plates of undiluted oyster homogenates had total counts that generally ranged between 100 and 200 colonies per plate (100 μl of homogenate per plate), making it impossible to enumerate the total number of colonies on a blot. In contrast, VVAP-positive colonies were easily detectable on blots even in this dense background, with counts corresponding to the number of *V. vulnificus* organisms inoculated.

TABLE 2. Enumeration of *V. vulnificus* and total heterotrophic bacteria in seeded, undiluted oyster homogenates and seawater samples

Inoculum[a]	Bacteria recovered (colonies/ml)			
	Oysters[b]		Seawater	
	VVAP[c]	Total[d]	VVAP	Total
1.4×10^4	1.1×10^4	1.8×10^4	2.0×10^4	2.1×10^4
1.4×10^3	7.8×10^2	2.5×10^3	1.4×10^3	2.0×10^3
1.4×10^2	1.8×10^2	1.3×10^3	1.0×10^2	1.0×10^3
1.4×10^1	1.0×10^1	1.7×10^3	0	9.0×10^2
1.4×10^0	0	1.4×10^3	0	1.0×10^3
0	0	1.4×10^3	ND[e]	ND

[a] Number of *V. vulnificus* M06-24 organisms added per milliliter of undiluted oyster homogenate or seawater sample.
[b] Results are means of two determinations of the number of bacteria per milliliter of undiluted oyster homogenate; 1 ml of undiluted oyster homogenate equals approximately 1 g of oyster meat.
[c] Number of *V. vulnificus* bacteria per milliliter as determined by hybridization with the *V. vulnificus* alkaline phosphatase probe.
[d] Number of heterotrophic bacteria as determined by growth on L agar.
[e] ND, not done.

DISCUSSION

In order to study the ecology of *V. vulnificus* and to evaluate the public health threat posed by this organism, a rapid, accurate method for the identification and enumeration of the organisms of this species within large populations of other bacteria is essential. Although the cytolysin gene of *V. vulnificus* has previously been shown to be an excellent marker for this species (6, 12, 13, 25), the present data are the first example of the use of a DNA probe to directly identify and enumerate *V. vulnificus* in environmental samples. Identification of *V. vulnificus* with the alkaline phosphatase-labeled probe showed exact correlation with identification based on the ^{32}P-labeled pCVD702 probe, API 20E, or fatty acid analysis, indicating that the 24-bp oligonucleotide retained both the sensitivity and specificity of the larger plasmid probe.

Previously described techniques for enumerating *V. vulnificus* require enrichment in alkaline peptone water and/or plating to selective media (4, 6, 13–15, 21, 22, 24). However, there can be problems with these methods. Enrichment involves competition with other, possibly faster growing organisms in a mixed population, and may result in overgrowth of unwanted bacteria (21). Detection of *V. vulnificus* in enrichment broths or in samples taken directly from the environment necessitates the use of selective media containing inhibitory compounds to eliminate background bacteria; however, freshly isolated environmental or clinical strains of *V. vulnificus* may also be sensitive to these additives (9, 13). Media used for selection usually contain indicators for sugar

fermentation that may not provide optimal differentiation of *V. vulnificus* from other organisms. For example, as with this study, another study (21) reported that 9 to 15% of *V. vulnificus* organisms were able to ferment sucrose; these strains would not be detected in studies that only examined sucrose-negative colonies on TCBS.

Final identification of *V. vulnificus* usually relies upon a battery of biochemical tests and/or immunoassays. These methods require the tedious subculture of numerous individual isolates, and the strain variability for many biochemical assays makes identification questionable unless a sufficient number of assays are done (14, 15). The cost and labor involved in this type of assessment can be prohibitive for many laboratories. Recent developments in immunological methodology for detection of this species have included more-simplified techniques such as a dipstick immune assay to detect flagellar antigens (17, 18) and an enzyme-linked immunoassay to detect an intracellular antigen (21). Monoclonal antibodies have proven highly specific (>99%) for identification of isolated *V. vulnificus*; however, their use still required enrichment or subculture of individual colonies from selective media to provide ample signal for detection. Immunoassay has failed at times to detect *V. vulnificus* in enrichment broths of oyster homogenates that yielded *V. vulnificus* when grown on selective medium, and it was postulated that this result may be due to competition with other bacteria in the assay (21). Immunological recognition of expressed phenotypes may also present problems if the trait is not constitutive or if the antigen shows cross-reactivity to other species (6, 13).

Polymerase chain reaction, based on amplification of the *V. vulnificus* cytolysin gene, offers the possibility of increased sensitivity; however, polymerase chain reaction amplification in complex substrates such as oyster homogenates may lack sensitivity because of inhibition of the *Taq* polymerase under these conditions. Seeded oyster homogenates required a 24-h incubation in enrichment broth for consistent amplification of extracted DNA (5); however, Brauns et al. (2) were able to detect directly viable but nonculturable *V. vulnificus* in seawater without enrichment at concentrations of $<10^5$ using primers from the cytolysin gene.

Using the alkaline phosphatase-labeled probe, *V. vulnificus* organisms were readily detectable in seawater from the Chesapeake Bay without enrichment at concentrations as low as 20 bacteria per ml. In our study, small numbers of bacteria were amplified by growth on nonselective solid medium, and the limits of detection were dependent upon the abilities of the bacteria to form colonies on agar and upon the number of background bacteria present. *V. vulnificus* was also enumerated in oyster homogenates at concentrations of 10^2 bacteria per ml, even on plates with a background of 100 to 1,000 colonies of heterotrophic bacteria per plate. Spread plates of oyster homogenates seeded with known concentrations of *V. vulnificus* yielded counts that closely approximated those of the inocula. This method did not detect bacteria at concentrations of <10/ml, nor can it identify bacteria that are viable but nonculturable; further efforts to increase sensitivity by combining the VVAP method with enrichment, filtration, or polymerase chain reaction techniques are being explored.

These data are the first assessment of the culturable *V. vulnificus* population in the estuarine environment without subculture to selective media or enrichment broth. In contrast, other studies have presented estimation of the *V. vulnificus* population either in terms of presence or absence in a sample (4, 6, 14, 15, 21) or in terms of the most probable number (22). Direct probing of colonies grown on nonselective medium should provide a sensitive means for evaluating other methods of enrichment or selection. The VVAP probe is also an improvement over the previously described plasmid probe in that the use of the synthetic oligonucleotide allows faster hybridization times, and more importantly, the replacement of radioisotopes with a phosphatase label will permit the application of this method to a variety of laboratory situations in which the use of ^{32}P is not feasible. As our environmental data are from only two time points at a single sampling site, further studies will be needed to accurately determine the distribution of *V. vulnificus* in Chesapeake Bay. However, our data suggest that the VVAP probe offers a reliable, rapid, and economical means of analyzing the public health threat posed by *V. vulnificus* in seafood and the estuarine environment.

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