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Real-Time PCR Assays for Quantification and Differentiation of *Vibrio vulnificus* Strains in Oysters and Water[▽]

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Vibrio vulnificus **is an autochthonous estuarine bacterium and a pathogen that is frequently transmitted via raw shellfish. Septicemia can occur within 24 h; however, isolation and confirmation from water and oysters require days. Real-time PCR assays were developed to detect and differentiate two 16S rRNA variants, types A and B, which were previously associated with environmental sources and clinical fatalities, respectively. Both assays could detect 10^2 to 10^3 *V. vulnificus* total cells in seeded estuarine water and in oyster homogenates. PCR assays on 11 reference *V. vulnificus* strains and 22 nontarget species gave expected results (type A or B for *V. vulnificus* and negative for nontarget species). The relationship between cell number and cycle threshold for the assays was linear ($R^2 = >0.93$). The type A/B ratio of Florida clinical isolates was compared to that of isolates from oysters harvested in Florida waters. This ratio was 19:17 in clinical isolates and 5:8 ($n = 26$) in oysters harvested from restricted sites with poor water quality but was 10:1 ($n = 22$) in oysters from permitted sites with good water quality. A substantial percentage of isolates from oysters (19.4%) were type AB (both primer sets amplified), but no isolates from overlying waters were type AB. The real-time PCR assays were sensitive, specific, and quantitative in water samples and could also differentiate the strains in oysters without requiring isolation of *V. vulnificus* and may therefore be useful for rapid detection of the pathogen in shellfish and water, as well as further investigation of its population dynamics.**

Vibrio vulnificus is a gram-negative bacterium that is autochthonous to warm estuarine waters and is frequently isolated from shellfish harvested in the Gulf of Mexico. *V. vulnificus* infections have been noted as the leading cause of food-related mortality in Florida (12). In the United States, nearly all foodborne infections result from the consumption of oysters collected from the Gulf of Mexico (5). Immunocompromised individuals and those with diseases causing increased iron levels in the body, such as liver disease or hemochromatosis, are at relatively high risk for development of primary septicemia, with a mortality rate of around 50% (6). There is also a risk of acute gastroenteritis due to the consumption of raw or undercooked seafood such as oysters (9). Infections can also occur due to trauma associated with handling contaminated seafood or the contact of open wounds with water containing *V. vulnificus*. Wound infections can become fatal or so severe that amputation is necessary to stop the spread of infection (17). Although the health of the host is a factor, it is not an absolute determinant of infection or clinical outcome (26).

Regulations for water quality in shellfish-harvesting areas in Florida rely on testing for indicator organisms, i.e., fecal coliforms (http://www.floridaaquaculture.com/SEAS/SEAS_intro.htm). For the most part, indicator bacteria have not been shown to correlate with the presence of pathogenic *Vibrio* spp. (13, 22, 27). At present, proposed risk assessment models rely on testing for the total *V. vulnificus* population (10). Currently accepted methods for isolation of *V. vulnificus* from seafood require plating on one of several selective-differential media (11, 14), followed by confirmation by biochemical or molecular tests (4, 11, 14, 15, 32). These methods require at least 24 h for completion and do not take into account the potential for variation in strain virulence.

Efforts to identify *V. vulnificus* virulence factors have met with various degrees of success. The capsular polysaccharide is present in nearly all *V. vulnificus* strains at the time of isolation, although translucent variants that are avirulent may arise during laboratory culture (28, 34). One study (33) showed that inactivation of the cytolysin gene did not affect the 50% lethal dose of virulent *V. vulnificus* strains. Sequence polymorphism of the 16S rRNA gene (3) was used to develop a restriction fragment polymorphism (RFLP) method to differentiate *V. vulnificus* strains (19). Typing of *V. vulnificus* isolates grouped environmental isolates in RFLP type A (94%), while the majority of those from clinical cases were RFLP type B (76%), as were 94.4% of the strains from clinical fatalities (19).

In this study, we developed primers for use in a SYBR green-based real-time PCR assay to detect and differentiate rRNA type A and B *V. vulnificus* without an isolation requirement.

Development of a rapid, cost-effective test for the relatively virulent strain(s) of this pathogen would aid in more accurate exposure assessment and perhaps more appropriate allocation of resources to mitigate the risk.

MATERIALS AND METHODS

Bacterial strains. *V. vulnificus* strains of clinical origin ($n = 36$) and nontarget isolates ($n = 22$) were obtained from the culture collection of the Food and Drug Administration (FDA) Gulf Coast Seafood Laboratory, from Daniel Lim (Uni-

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versity of South Florida, Tampa), from A. Cannons and R. Baker (Florida Department of Health [FDOH]), from the Centers for Disease Control and Prevention (CDC), and from the American Type Culture Collection (ATCC). The 11 reference strains used to establish agreement among the typing methods were clinical or environmental strains obtained from the FDA (10 strains) and from ATCC (27562). Environmental strains ($n = 82$) were collected from the Guana-Tolomato-Matanzas National Estuarine Research Reserve near St. Augustine on the east coast of Florida during four sampling events, from Tampa Bay during two sampling events, and from Apalachicola, FL, during one sampling event. Samples collected from St. Augustine and Tampa Bay were collected from nonpermitted oyster-harvesting areas when the water temperature was generally >24°C. Oyster samples collected in Apalachicola were harvested by a commercial shellfishing company from permitted oyster-harvesting areas in August 2005, when water temperatures were also >24°C. Oyster homogenates were prepared from oysters diluted 1:10 (wt/wt) in alkaline peptone water (APW). All putative *V. vulnificus* isolates were confirmed by PCR by using the *vvhA* gene (15).

Specificity assays were conducted on 22 nontarget bacterial species, including closely related vibrios (*V. alginolyticus* ATCC 51160; *V. cholerae* El Tor, O1, and ATCC 25780; *V. parahaemolyticus* ATCC 10290 and ATCC 49398; and *Aeromonas hydrophila* ATCC 7965 and ATCC 14715), as well as members of the coliform groups commonly found in marine and estuarine waters (*Citrobacter freundii* ATCC 8090, *Enterobacter agglomerans* ATCC 27981, *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Enterococcus mundtii*, *Klebsiella ozaenae* ATCC 29019, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 15442, *Serratia marcescens* ATCC 14756, *Weisella confusa* ATCC14434, and *Yersinia enterocolitica* ATCC 9610).

Preparation of pure cultures. For specificity, sensitivity, and typing assays, *Vibrio* spp. and other isolates were grown in 25 ml brain heart infusion broth supplemented with 0.5% NaCl for 18 to 24 h at 25°C with shaking. For sensitivity assays, *V. vulnificus* ATCC 33814 and vPvMH1003-12 were concentrated by centrifugation at 14,500 × *g* for 10 min. The supernatant was removed, and the pellet was washed twice with 2.0 ml of 0.85% NaCl before being resuspended in 4.0 ml of 0.85% NaCl. Serial dilutions were then made in 0.85% NaCl or in an alternative matrix, as noted. Alternative matrices included filter-sterilized Instant Ocean (20‰; Aquarium Systems Inc., Mentor, OH) and unsterilized water collected from Tampa Bay, FL (pH 7.96, salinity of 28 ‰, and temperature of 21°C at time of collection). The concentration of cells was determined by using total direct microscopic counts (see below), and consistent volumes of successive dilutions were then used as the templates in real-time PCR assays. DNA was extracted only for the specificity and typing assays with a DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions and quantified with a Beckman DU640 spectrophotometer (Beckman Coulter) with a 2:7 dilution in 10 mM Tris-HCl (pH 8.5).

Total cell counts. Dilution series were made in 0.85% NaCl (or appropriate medium) as described above for direct fluorescence microscopic counts. DAPI (4′,6′-diamidino-2-phenylindole) staining was performed essentially as previously described (23), with 1 μl of DAPI (1 mg · ml^{-1}) to stain 1-ml samples. Each dilution was filtered through a 0.2-μm GTBP Isopore membrane filter (Millipore, Burlington, MA) and observed under a Nikon Diaphot inverted fluorescence microscope. The number of cells in a 20-square area (0.002 mm^2) of the ocular grid was recorded for five different fields of view, and cell concentrations were calculated.

Primer development. Sequences for two 16S rRNA variants of *V. vulnificus* identified by Aznar et al. (3) were obtained from GenBank (accession numbers X67333 and X76334). The two sequences were aligned with GeneDoc (18) to identify the positions of the 17 base pair differences. Forward primer sequences were adapted from probes previously designed by Michael Vickery et al. (30); the type A forward primer was VvAF1 (5′-CAT GAT AGC TTC GGC TCA A-3′), and type B forward primer was VvBF1 (5′-GCC TAC GGG CCA AAG AGG-3′). The positions of the reverse primers (VvAR1 [5′-CAG CAC TCC TTC CAC CAT CAC-3′] and VvBR1 [5′-GTC GCC TCT GCG TCC AC-3′]) were chosen with the aid of PrimerQuest (24) to allow easily differentiable PCR products (type A = 245 bp; type B = 841 bp) while capitalizing on as many of the available base pair variations as possible. Initially, the specificity of the primers was assessed with BLAST (National Center for Biotechnology Information) (2), which showed 100% similarity for each primer set only with *V. vulnificus* sequences. PCR conditions were optimized and then adapted for use with SYBR green (Roche Diagnostics, Germany) in a real-time PCR assay with the LightCycler 2.0 (Roche).

Real-time PCR protocol. Real-time PCR assay mixtures of 20 μl contained 2 mM $MgCl_2$, 0.5 μM each primer, 2 μl of LightCycler FastStart DNA Master SYBR green I reaction mix (Roche Diagnostics, Germany), and the PCR template (whole cells or extracted DNA). When purified DNA was used as the template (for specificity and typing analysis), 2 μl of template solution containing 4 to 20 ng of DNA was added to each reaction mixture. Whole cells were used for sensitivity analysis. Positive and negative controls were included for each set of reaction mixtures. For type A primers, an environmental *V. vulnificus* isolate (vPvMH1003-12) was used, and for type B primers, *V. vulnificus* ATCC 33814 was used. An initial 10-min denaturation step at 95°C was followed by a two-step amplification program of 35 cycles of 95°C for 15 s and 74°C for 35 s. Triplicate reaction mixtures were prepared to confirm the reproducibility of the results. PCR products for both primer sets were also sequenced with the GenomeLab DTCS Quick Start kit (Beckman Coulter, Fullerton, CA) to ensure that the correct amplicons were produced.

RFLP confirmation. Restriction fragment length polymorphism (RFLP) analysis was performed on PCR products from 11 reference *V. vulnificus* strains with AluI and HaeIII as previously described (19) in order to validate the PCR typing method.

Sensitivity. *V. vulnificus* concentrations were determined by direct microscopic counts. Cells were diluted in 0.85% NaCl in order to add from 10^6 cells to 1 cell to each PCR mixture. Reaction mixtures were also prepared with a mixture of equal numbers of cells of *V. vulnificus* ATCC 33814 and vPvMH1003-12. To test primer sensitivity in more natural matrices, dilution series were also made with filter-sterilized 20‰ Instant Ocean (Aquarium Systems Inc., Mentor, OH) and estuarine water (unsterilized water from Tampa Bay) as described above.

Type A and type B control *V. vulnificus* cells were inoculated into 300 ml of water collected from Tampa Bay (salinity, ~28‰) to a final concentration of ~160 cells/100 ml. The estuarine water was assayed for *V. vulnificus* prior to the experiment and did not contain detectable levels of the bacterium. The seeded water was filtered through a 0.45-μm-pore-size nitrocellulose filter (Osmonics Inc., Westborough, MA). Genomic DNA was extracted from the filter with the UltraClean Soil DNA kit (Mo Bio Laboratories Inc., Solana Beach, CA). The final elution volume was 50 μl (6,000-fold nominal concentration factor). Two microliters of the extracted DNA was used as the template in triplicate real-time PCR assays as described above.

The sensitivity of the assays in homogenized oyster tissue was tested by seeding 2 μl of oyster homogenate (see below), which had not previously shown any amplification with the primer sets, with 1 to 10^7 cells of type A or type B control *V. vulnificus*. The seeded homogenate was then directly analyzed by real-time PCR.

PCR detection of native *V. vulnificus* in oyster homogenates. Tissue from a single oyster collected from Tampa Bay was diluted 1:10 (wt/wt) in APW (14). Homogenate was made by blending at high speed for 90 s. A 25-ml aliquot was pipetted into two centrifuge tubes for enrichment at 37°C for 4 and 24 h. Enrichments and oyster homogenate were immediately cultured (see below), and aliquots were stored at −20°C for later testing by the real-time PCR.

To determine the concentrations of culturable *V. vulnificus* naturally present in oyster homogenates, *V. vulnificus* was isolated from initial homogenates and dilutions of enriched homogenates by spreading 0.1 ml onto *V. vulnificus* agar (VVA) (14). VVA plates were incubated overnight at 37°C in order to determine the presence of type A and/or type B *V. vulnificus* in each oyster. Cellobiose-fermenting colonies from VVA plates were transferred individually to 96-well microtiter plates containing 180 μl T_1N_1 agar and stored at room temperature for further analysis. The same set of VVA plates was then probed according to Wright et al. (32) with hybridization at 56°C. The colonies that gave positive reactions with the probe were then typed by real-time PCR.

The original oyster homogenate and enriched homogenates (4-h and 24-h enrichments) were prepared for PCR assays by dilution at 1:10 (vol/vol) in 0.85% NaCl. Two milliliters of each diluted homogenate was boiled for 15 min to lyse cells and centrifuged at 16,000 × *g* to pellet cellular debris. Two microliters of the supernatant was the template for each PCR. To monitor for possible inhibition of amplification from oyster homogenates, positive control reaction mixtures were made which included 10 to 50 ng of *V. vulnificus* DNA (type A and type B).

Statistical analysis. Linear regression of the cycle threshold (C_t value) versus the seeded cell numbers (enumerated by direct microscope count) was conducted in Microsoft Excel. A contingency table and a chi-square test (GraphPad InStat v. 3.00) were used to compare the A/B/AB ratios of *V. vulnificus* isolates.

RESULTS

Both primer sets performed as expected, as each amplified the 16S rRNA gene from the corresponding type of *V. vulnificus* and no product was produced when the template was the

TABLE 1. *V. vulnificus* strains, origins, and types[a]

Strain	Source	PCR result
ATCC 27562 [d]	Clinical, FL	A
9031-96[b,d]	FDA, clinical, FL	A
4933[c]	FDOH, clinical, FL	A
4939[b]	FDOH, clinical, FL	A
5011	FDOH, clinical, FL	A
5192[c]	FDOH, clinical, FL	A
6288[b]	FDOH, clinical, FL	A
6325[c]	FDOH, clinical, FL	A
6689	FDOH, clinical, FL	A
CBD 113	FDOH, clinical, FL	A
1497-82	CDC, clinical, Romania	A
1498-82	CDC, clinical, Romania	A
2415-01	CDC, clinical, TN	A
2430-01	CDC, clinical, FL	A
2432-02	CDC, clinical, LA	A
2438-02	CDC, clinical, CO	A
2448-03	CDC, clinical, VA	A
2428-06	CDC, clinical, LA	A
2432-06	CDC, clinical, LA	A
ATCC 33814	Clinical, FL	B
9053-96[b,d]	FDA, clinical, TX	B
9067-96[b,d]	FDA, clinical, TX	B
6130[b]	FDOH, clinical, FL	B
6283[c]	FDOH, clinical, FL	B
6434[c]	FDOH, clinical, FL	B
4263	FDOH, clinical, FL	B
4265[b]	FDOH, clinical, FL	B
4350	FDOH, clinical, FL	B
4351	FDOH, clinical, FL	B
4352	FDOH, clinical, FL	B
5274[c]	FDOH, clinical, FL	B
T&CH 83104-MT#1	FDOH, clinical, FL	B
T&CH 83104-MT#2	FDOH, clinical, FL	B
2428-01	CDC, clinical, UT	B
2431-01	CDC, clinical, New York City	B
2450-06	CDC, clinical, HI	B

[a] Type A isolates obtained from clinical samples in Florida are in bold.
[b] Infection through ingestion of contaminated oysters.
[c] Wound infection.
[d] Included in reference strain set.

opposite *V. vulnificus* type. Agarose gel electrophoresis of PCR products indicated that amplicons were of the expected sizes (285 bp for type A and 841 bp for type B) and had average melting temperatures of 89.0 ± 0.3°C for the products of the type A and type B primer sets. BLAST (2) analysis showed that the DNA sequence of the PCR product produced by each primer set was most closely related to that of its respective *V. vulnificus* strain in GenBank. Multiplex assays with the A and B primers in the same reaction mixture were not extensively explored due to the possibility of amplification of *V. parahaemolyticus* DNA in a multiplex format.

The specificity of each primer set was tested against 22 nontarget organisms which included closely related *Vibrio* spp. (see Materials and Methods), as well as clinical and environmental *V. vulnificus* strains (Tables 1 and 2). No amplification was observed with any of the nontarget bacterial isolates tested. DNA from each *V. vulnificus* isolate was amplified, confirming it as type A, type B, or, in some cases, type AB (Tables 1 and 2); the latter denotes positive results with both the A and B primer sets in separate reaction mixtures.

TABLE 2. Genotypes of *V. vulnificus* isolates from oysters, water, and clinical sources

Isolate source	No. of isolates from oysters or water[b]			
	Type A	Type B	Type AB	Total
Tampa Bay	8, 5	13, 4	5, 0	26, 9
St. Augustine	2, 4	3, 2	0, 0	5, 6
Apalachicola	20, 0	2, 0	8, 0	30, 0
Previously typed[a]	5, 0	1, 0	0, 0	6, 0
Total	35, 9	19, 6	13, 0	67, 15
Clinical	19	17	0	36
Total	63	42	13	118

[a] References 19 and 30.
[b] For paired values, the first number indicates isolates from oysters and the second number indicates isolates from water.

RFLP confirmation. Of the 11 reference strains that were also typed by RFLP with both restriction enzymes, 9 returned the same type with all of the methods. For two isolates, the HaeIII results agreed with the real-time PCR type; however, the AluI pattern was a mixture of the banding patterns expected for type A and type B, indicating a type AB result, which has been seen previously (30).

Sensitivity. Assay sensitivity was determined as the minimum number of seeded *V. vulnificus* cells required for amplification in three replicate PCRs. Cell numbers in pure cultures were assessed by direct microscopic counts, providing a more accurate and sensitive estimate of total cell concentrations than would culturable counts. In 0.85% NaCl with and without an equal number of nontarget type B cells added, the VvAF1/VvAR1 primer set (type A) was able to quantify 10^2 to 10^6 cells of *V. vulnificus* vPvMH1003-12. In these same matrices and treatments, primer set VvBR1/VvBF1 (type B) was consistently able to quantify 10 to 10^6 *V. vulnificus* ATCC 33814 cells per PCR assay. Linear regression showed an excellent correlation between cell number and C_t (average $R^2 = 0.97 \pm 0.04$ for both assays). The assay sensitivity was 10^3 cells for the type A primer set and 10^2 cells for the type B primer set in 20‰ Instant Ocean ($R^2 = \geq 0.93$) or unsterilized estuarine water ($R^2 = \geq 0.98$). This demonstrates the potential usefulness of these assays in quantifying the two *V. vulnificus* types in estuarine waters. In our laboratory, the comparison of culturable to direct microscopic cell counts for an 18-h culture in brain heart infusion broth plus 0.5% NaCl showed that culturable counts were $\sim 10^2$ lower than direct counts (data not shown). In terms of culturable cells, this increases the apparent sensitivity in estuarine waters to 10 CFU per reaction mixture for the type A assay and 1 CFU per reaction mixture for the type B assay.

To demonstrate that cell concentrations representative of levels found in natural environments could be detected by the PCR assays, *V. vulnificus* cells were seeded into 300 ml of estuarine water and subsequently concentrated by membrane filtration and detected by real-time PCR. Amplification of samples concentrated from estuarine waters seeded with 160 cells $\cdot$ 100 ml^{-1} was observed in triplicate reaction mixtures (each representing analysis of a sample of ~12 ml before concentration) containing ~20 cells of either type A or type B *V. vulnificus*. The C_t values obtained in this experiment were

consistent with standard curves for both typing assays, which estimated ~17.4 and ~4 cells per reaction mixture for types A and B, respectively.

In seeded oyster homogenates, the type A primer set was able to detect 10^3 to 10^6 cells per reaction mixture while the type B primers could detect 10^2 to 10^6 cells per reaction mixture.

PCR detection of native *V. vulnificus* in oyster homogenates. Amplification from an unseeded oyster homogenate was achieved with the type B primer set after enrichment in APW for 24 h. Before enrichment, the concentration of culturable *V. vulnificus* in the homogenate, which was derived from a single oyster, was 130 CFU $\cdot$ g^{-1} oyster tissue, or 13 CFU $\cdot$ ml^{-1} in the 1:10 homogenate. This enriched homogenate contained 1.2×10^7 CFU $\cdot$ ml^{-1} *V. vulnificus* or 2.4×10^4 CFU per PCR assay. In contrast, no amplification with type B primers was detected from the homogenate without enrichment or with enrichment for 4 h (which contained 2.5×10^3 CFU *V. vulnificus* $\cdot$ ml^{-1} enrichment). Furthermore, none of the reaction mixtures with the type A primers yielded amplification.

Typing of the naturally occurring *V. vulnificus* strains isolated from the oyster homogenate yielded 100% type B (n = 9), which explains the failure of amplification with type A primers. The lack of amplification by type B primers observed in the unenriched oyster homogenate and 4-h-enriched oyster homogenates was due to the relatively low number of naturally occurring *V. vulnificus* bacteria in this oyster. The oyster homogenate prior to enrichment contained 13 CFU $\cdot$ ml^{-1} *V. vulnificus* or 0.026 culturable cells per PCR. After a 4-h enrichment, this increased to five culturable cells per PCR.

Typing of clinical and environmental isolates. Thirty-six clinical isolates were typed in this study (Table 1). Fifty-three percent of these isolates were type A, and 47% were type B. Of the 32 clinical isolates not typed in previous studies (19, 30), 17 (53%) were type A and 15 (47%) were type B. Chi-square analysis showed no significant difference between the relative frequencies of the types and the distribution that would be expected by chance (P = 0.8025); i.e., neither type made up a greater proportion of the clinical isolates. The route of infection for 13 of the clinical isolates not typed in previous studies was known. Six were the result of wound infections, and seven were from oyster consumption (Table 1).

Seventy-six environmental isolates from water and oysters were typed that were not typed in previous studies. All water samples (n = 15) were from Tampa Bay or St. Augustine, where shellfishing is prohibited due to poor water quality in both areas. Sixty percent of the 15 isolates from prohibited shellfishing waters were type A, and 40% were type B (Table 2). Chi-square analysis showed no significant difference in the observed frequency of isolation of each type from water and that expected by chance (P = 0.7144).

Sixty-seven isolates from oysters were typed (Table 2). In oysters from Tampa and St. Augustine, the majority of isolates were type B (the A/B/AB ratio was 10:16:5; n = 31). To determine whether the frequency of the types isolated from oysters was different in prohibited versus permitted shellfishing waters, chi-square analysis was performed and the frequencies were found to differ significantly (chi-square = 14.902, df = 2, P = 0.0006). Type B *V. vulnificus* was more frequently isolated from prohibited waters than from permitted waters (prohibited/permitted ratio = 16:2). In contrast, type A *V. vulnificus* was more frequently isolated from oysters in permitted waters (prohibited/permitted ratio = 10:20). Interestingly, type AB isolates were isolated at about the same frequency from oysters harvested from prohibited versus permitted waters (prohibited/permitted ratio = 5:8). Although type AB strains complicate the estimation of the relative frequency and numbers of *V. vulnificus* strains, they are in the minority in *V. vulnificus* populations (e.g., 21% of the isolates tested by Vickery et al. [30]).

DISCUSSION

The primers developed were able to amplify DNA from 100% of the *V. vulnificus* isolates, identifying them as type A, type B, or type AB. RFLP analysis of 11 previously typed *V. vulnificus* isolates (19, 30) showed 82% agreement with the real-time PCR assay types, determining the isolates to be type A, B, or AB. The two discrepancies occurred when the AluI enzyme of the RFLP assay specified type AB while the HaeIII RFLP results and the PCR assays specified type A. Other studies have also documented the presence of type AB isolates (25, 30) due to amplification of genetic material from one isolate by primers or probes for both types A and B. *V. vulnificus* YJ016 contains nine rRNA operons (8), which are all type B according to their sequences in GenBank. However, cloning and sequencing of 492 bp of the 16S rRNA gene from one type AB isolate showed the presence of type A and type B 16S rRNA genes (30). Heterogeneity between multiple 16S rRNA operon copies has also been observed within the same strain of several related bacteria, including *V. parahaemolyticus* and *V. cholerae* (1).

Sensitivity. *V. vulnificus* could be detected at 160 cells/100 ml (1.6 cells $\cdot$ ml^{-1}) in a water sample. Other studies have developed methods to detect *V. vulnificus* in water samples by real-time PCR targeting genes such as the cytolysin/hemolysin gene and *toxR* (4, 20, 21, 29, 31). The sensitivities of these methods ranged from the equivalent of 10^1 to 10^2 CFU $\cdot$ ml^{-1} in seeded water samples (4, 21, 29) when using extracted DNA as the template. The sensitivity of our assay in water samples was greater than those previously reported. The assays are not as sensitive or rapid in oyster homogenates as they are in water (13 CFU $\cdot$ ml^{-1} after 24 h enrichment), but they do have the advantage of yielding information on *V. vulnificus* type, as well as presence. The real-time PCR for the 4-h enrichment, which should have contained enough cells for detection, may have returned a false-negative result due to the speed at which the samples were centrifuged ($16{,}000 \times g$). Analysis of the fractionation of the PCR product between the supernatant and pellet from replicate experiments showed that between 31% and 59% of the total DNA amplified appeared in the pellet (data not shown).

A/B ratios and geographic distribution. The A/B ratio observed for clinical isolates analyzed in this study was almost even at 19:17. These results contrast with those of previous studies, in which only 24% (8 of 34) of the clinical isolates from oysters originating in the United States were type A or type AB (19, 30). However, six of the eight clinical cases caused by type A or AB *V. vulnificus* in the previous studies were contracted through the consumption of oysters harvested in Florida. In our study, the proportion of type A clinical isolates from Flor-

ida was 11:12 (48% type A). The combined results of these studies (this study and references 19 and 30) indicate that Florida clinical isolates are much more likely to be type A than are clinical isolates from other states, suggesting the possibility of geographical variation in the population structure of *V. vulnificus*. A recent study explored relationships among *V. vulnificus* strains based on four distinct genetic measures (rep-PCR, 16S A/B, capsular polysaccharide operon group, and RAPD profile) (7). Type A and AB clinical *V. vulnificus* strains were grouped into two distinct clusters (II and III) by rep-PCR patterns with other oyster isolates, while the type B clinical isolates tended to be grouped separately into clusters I, IV, and VII, supporting the hypothesis that genetically distinct subpopulations of *V. vulnificus* exist.

In contrast to results for clinical isolates, the A/B ratios observed for environmental isolates from this study were in agreement with previous studies (19, 30); type A strains were the majority of those isolated from oysters collected from permitted harvest areas. Note, however, that the frequency of type B *V. vulnificus* in oysters from prohibited areas was much greater than the frequency of type B strains in oysters from permitted waters. The differences in strain distribution should be interpreted in light of the different sampling strategies employed in the studies; most of the environmental *V. vulnificus* strains typed in the previous studies (19, 30) were isolated from oysters collected from conditionally approved and commercially harvested sites, while the majority of the isolates in our study were isolated from oysters in restricted areas with lower water quality. Furthermore, the majority of the environmental isolates in this study were collected between August and November, while in previous studies comparing *V. vulnificus* type and isolate source (19, 30), isolates were collected throughout the year. A study conducted in Galveston Bay, TX, noted seasonal variation in *V. vulnificus* A/B ratios, as type B isolates were observed frequently in the warmer summer months but not in cooler months (16). These data indicate that factors such as season and water quality may influence A/B genotype ratios and should be further explored. The typing of environmental and clinical *V. vulnificus* isolates from a broad geographic range by a sampling strategy that accounts for seasonal variation and with a variety of molecular tools is necessary to better understand the effects of environmental and genetic variation on *V. vulnificus* infection rates.

These rapid A/B PCR assays (~90 min in water samples) provide a quantitative "piece of the puzzle" of the population structure of *V. vulnificus* in coastal waters. These assays do not require culturing and confirming individual isolates, which is an added benefit over recently published A/B typing methods (19, 30). This assay could prove useful in routine monitoring of shellfish-harvesting waters, which may in turn allow timely warning for consumers.

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REFERENCES

1. **Acinas, S. G., L. A. Marcelino, V. Klepac-Ceraj, and M. F. Polz.** 2004. Divergence and redundancy of 16S rRNA sequences in genomes with multiple *rrn* operons. J. Bacteriol. **186:**2629–2635.
2. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Aznar, R., W. Ludwig, R. I. Amann, and K.-H. Schleifer.** 1994. Sequence determination of rRNA genes of pathogenic *Vibrio* species and whole-cell identification of *Vibrio vulnificus* with rRNA-targeted oligonucleotide probes. Int. J. Syst. Bacteriol. **44:**330–337.
4. **Campbell, M. S., and A. C. Wright.** 2003. Real-time PCR analysis of *Vibrio vulnificus* from oysters. Appl. Environ. Microbiol. **69:**7137–7144.
5. **Centers for Disease Control and Prevention.** 1996. *Vibrio vulnificus* infections associated with eating raw oysters—Los Angeles, 1996. Morb. Mortal. Wkly. Rep. **45:**621–624.
6. **Centers for Disease Control and Prevention.** 1993. *Vibrio vulnificus* infections associated with raw oyster consumption—Florida, 1981–1992. Morb. Mortal. Wkly. Rep. **42:**405–407.
7. **Chatzidaki-Livanis, M., M. A. Hubbard, K. V. Gordon, V. J. Harwood, and A. C. Wright.** 2006. Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. Appl. Environ. Microbiol. **72:**6136–6141.
8. **Chen, C. Y., K. M. Wu, Y. C. Chang, C. H. Chang, H. C. Tsai, T. L. Liao, Y. M. Liu, H. J. Chen, A. B. Shen, J. C. Li, T. L. Su, C. P. Shao, C. T. Lee, L. I. Hor, and S. F. Tsai.** 2003. Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. Genome Res. **13:**2577–2587.
9. **DePaola, A., G. M. Capers, and D. Alexander.** 1994. Densities of *Vibrio vulnificus* in the intestines of fish from the U.S. Gulf Coast. Appl. Environ. Microbiol. **60:**984–988.
10. **Food and Agriculture Organization of the United Nations.** 2002. Risk assessment of Campylobacter spp. in broiler chickens and Vibrio spp. in seafood. Food and Agriculture Organization of the United Nations, Rome, Italy. http://www.who.int/foodsafety/publications/micro/en/aug2002.pdf.
11. **Harwood, V. J., J. P. Gandhi, and A. C. Wright.** 2004. Methods for isolation and confirmation of *Vibrio vulnificus* from oysters and environmental sources: a review. J. Microbiol. Methods **59:**301–316.
12. **Jackson, J. K., R. L. Murphree, and M. L. Tamplin.** 1997. Evidence that mortality from *Vibrio vulnificus* infection results from single strains among heterogeneous populations in shellfish. J. Clin. Microbiol. **35:**2098–2101.
13. **Kaper, J., H. Lockman, R. R. Colwell, and S. W. Joseph.** 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. Appl. Environ. Microbiol. **37:**91–103.
14. **Kaysner, C. A., and A. DePaola.** 2004. *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and other *Vibrio* spp., chapter 9. *In* Bacteriological analytical manual online, 8th ed., revision A, 1998. Substantially rewritten and revised May 2004. http://www.cfsan.fda.gov/~ebam/bam-9.html.
15. **Lee, S. E., S. Y. Kim, S. J. Kim, H. S. Kim, J. H. Shin, S. H. Choi, S. S. Chung, and J. H. Rhee.** 1998. Direct identification of *Vibrio vulnificus* in clinical specimens by nested PCR. J. Clin. Microbiol. **36:**2887–2892.
16. **Lin, M., and J. R. Schwarz.** 2003. Seasonal shifts in population structure of *Vibrio vulnificus* in an estuarine environment as revealed by partial 16S ribosomal DNA sequencing. FEMS Microbiol. Ecol. **45:**23–27.
17. **Linkous, D. A., and J. D. Oliver.** 1999. Pathogenesis of *Vibrio vulnificus*. FEMS Microbiol. Lett. **174:**207–214.
18. **Nicholas, K. B., H. B. J. Nicholas, and D. W. I. Deerfield.** 1997. GeneDoc: analysis and visualization of genetic variation. EMBnet.news **4:**1–4. http://www.es.embnet.org/embnet_common/embnet.news/pdf/emnn42.pdf.
19. **Nilsson, W. B., R. N. Paranjype, A. DePaola, and M. S. Strom.** 2003. Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. J. Clin. Microbiol. **41:**442–446.
20. **Panicker, G., and A. K. Bej.** 2005. Real-time PCR detection of *Vibrio vulnificus* in oysters: comparison of oligonucleotide primers and probes targeting *vvhA*. Appl. Environ. Microbiol. **71:**5702–5709.
21. **Panicker, G., M. L. Myers, and A. K. Bej.** 2004. Rapid detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water by real-time PCR. Appl. Environ. Microbiol. **70:**498–507.
22. **Pérez-Rosas, N., and T. C. Hazen.** 1989. In situ survival of *Vibrio cholerae* and *Escherichia coli* in a tropical rain forest watershed. Appl. Environ. Microbiol. **55:**495–499.
23. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:**943–948.
24. **Rozen, S., and H. J. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers, p. 365–386. *In* S. Misener and S. A. Krawetz (ed.), Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, NJ.
25. **Senoh, M., S. Miyoshi, K. Okamoto, B. Fouz, C. Amaro, and S. Shinoda.** 2005. The cytotoxin-hemolysin genes of human and eel pathogenic *Vibrio*

vulnificus strains: comparison of nucleotide sequences and application to the genetic grouping. Microbiol. Immunol. **49:**513–519.

26. **Shapiro, R. L., S. Altekruse, L. Hutwagner, R. Bishop, R. Hammond, S. Wilson, B. Ray, S. Thompson, R. V. Tauxe, and P. M. Griffin.** 1998. The role of Gulf Coast oysters harvested in warmer months in *Vibrio vulnificus* infections in the United States, 1988–1996. J. Infect. Dis. **178:**752–759.
27. **Shiaris, M. P., A. C. Rex, G. W. Pettibone, K. Keay, P. McManus, M. A. Rex, J. Ebersole, and E. Gallagher.** 1987. Distribution of indicator bacteria and *Vibrio parahaemolyticus* in sewage-polluted intertidal sediments. Appl. Environ. Microbiol. **53:**1756–1761.
28. **Simpson, L. M., V. K. White, S. F. Zane, and J. D. Oliver.** 1987. Correlation between virulence and colony morphology in *Vibrio vulnificus*. Infect. Immun. **55:**269–272.
29. **Takahashi, H., Y. Hara-Kudo, J. Miyasaka, S. Kumagai, and H. Konuma.** 2005. Development of a quantitative real-time polymerase chain reaction targeted to the *toxR* for detection of *Vibrio vulnificus*. J. Microbiol. Methods **61:**77–85.
30. **Vickery, M. C., W. B. Nilsson, M. S. Strom, J. L. Nordstrom, and A. DePaola.** 2007. A real-time PCR assay for the rapid determination of 16S rRNA genotype in *Vibrio vulnificus*. J. Microbiol. Methods **68:**376–384.
31. **Wang, S., and R. E. Levin.** 2006. Rapid quantification of *Vibrio vulnificus* in clams (*Protochaca staminea*) using real-time PCR. Food Microbiol. **23:**757–761.
32. **Wright, A. C., G. A. Miceli, W. L. Landry, J. B. Christy, W. D. Watkins, and J. G. Morris, Jr.** 1993. Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. Appl. Environ. Microbiol. **59:**541–546.
33. **Wright, A. C., and J. G. Morris, Jr.** 1991. The extracellular cytolysin of *Vibrio vulnificus*: inactivation and relationship to virulence in mice. Infect. Immun. **59:**192–197.
34. **Wright, A. C., L. M. Simpson, J. D. Oliver, and J. G. Morris, Jr.** 1990. Phenotypic evaluation of acapsular transposon mutants of *Vibrio vulnificus*. Infect. Immun. **58:**1769–1773.