APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2007, p. 7477–7481 Vol. 73, No. 22
0099-2240/07/$08.00+0 doi:10.1128/AEM.01118-07

Evaluation of Postharvest-Processed Oysters by Using PCR-Based Most-Probable-Number Enumeration of *Vibrio vulnificus* Bacteria[▽]

Anita C. Wright,* Victor Garrido, Georgia Debuex, Melissa Farrell-Evans, Archana A. Mudbidri, and W. Steven Otwell

University of Florida, Department of Food Science and Human Nutrition, P.O. Box 110370, Gainesville, Florida 32611

Received 18 May 2007/Accepted 18 September 2007

Postharvest processing (PHP) is used to reduce levels of *Vibrio vulnificus* in oysters, but process validation is labor-intensive and expensive. Therefore, quantitative PCR was evaluated as a rapid confirmation method for most-probable-number enumeration (QPCR-MPN) of *V. vulnificus* bacteria in PHP oysters. QPCR-MPN showed excellent correlation ($R^2 = 0.97$) with standard MPN and increased assay sensitivity and efficiency.

Vibrio vulnificus can cause life-threatening, systemic disease (2, 9, 14) that is associated with the consumption of raw oysters. The bacterium is distributed throughout temperate estuaries worldwide (6, 7, 17, 24, 25), and environmental conditions of warmer water temperature and lower salinity favor its growth in molluscan shellfish (5, 13, 16, 21, 23, 25). Warning labels on oyster products and educational programs have not been effective in reducing disease mortality rates for at-risk individuals with underlying diseases, such as cirrhosis, hemochromatosis (iron overload), diabetes, or immune system dysfunction (8). Therefore, the FDA and the Interstate Shellfish Sanitation Conference (ISSC) have mandated postharvest processing (PHP) of oysters harvested from Gulf Coast states in order to reduce *V. vulnificus* infections (11). Application of PHP methodology requires validation and verification in order to ensure that the process will substantially reduce numbers of *V. vulnificus* bacteria to levels that are below the predicted threshold for disease. Validation trials for PHP are labor-intensive and cost-prohibitive (10, 12), and improved protocols for industry compliance and for risk assessment of *V. vulnificus* in PHP oysters are urgently needed.

The standard method for validating PHP requires that three independent lots of oysters meet the specification of <30 most probable numbers (MPN)/g of *V. vulnificus* by using the geometric mean of 10 samples/lot. Levels of *V. vulnificus* bacteria in oysters are enumerated by MPN endpoint titration of replicate samples in enrichment broth cultures (12), and species-specific growth is determined by isolating typical *V. vulnificus* colonies on selective medium, with subsequent confirmation by DNA probe (26). The present study evaluated a real-time quantitative PCR (QPCR) assay for detection of *V. vulnificus* growth in MPN enrichment cultures (QPCR-MPN). QPCR increases assay throughput by using automated species-specific confirmation, which is not available with standard PCR. Also, the limit of detection for direct QPCR enumeration of *V. vulnificus* bacteria in oysters without enrichment is generally 100 CFU/g, but QPCR-MPN assays generally increase the sensitivity of detection to 1 bacterium/g after enrichment (1, 18, 19, 20) and permit detection of *V. vulnificus* at levels (30 CFU/g) required for validation protocols.

Field trials were conducted to assess application of QPCR-MPN to oyster PHP validation, as application of QPCR-MPN to enumerate *V. vulnificus* bacteria in PHP oysters has not been examined previously and merits further scrutiny. For example, large numbers of dead bacteria may accumulate in the oyster product as a consequence of PHP and could provide a DNA template for false-positive amplification of nonviable bacteria. The present study used immersion of oysters in liquid nitrogen, followed by extended frozen storage at −20°C as an ultralow-temperature PHP for oysters. Samples were examined before, during, and after exposure to PHP to provide a side-by-side comparison of the standard MPN protocol to QPCR-MPN confirmation. Results support the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster PHP.

TaqMan versus SYBR green I QPCR assays for *V. vulnificus*. We previously reported a TaqMan QPCR assay that targeted the *V. vulnificus vvhA* gene, which encodes a hemolytic cytolysin. Prior examination of target ($n = 28$) and nontarget ($n = 22$) strains showed that *V. vulnificus* QPCR was 100% specific and detected about 10^2 CFU/g in oysters (4). The assay was modified in the present study to use boiling lysis rather than Qiagen tissue kits for DNA extraction. SYBR green I dye detection was compared to TaqMan in order to reduce assay cost and simplify the protocol for subsequent MPN applications. The type of thermocycler (Cepheid) and addition of SmartMix beads (Cepheid) also differed from the prior assay, which used an Applied Biosystems thermocycler and reagents. For boiling lysis, cultures (1 ml) were centrifuged (15,000 × *g*, 10 min), resuspended in phosphate-buffered saline (PBS) (1 ml), boiled for 10 min, and subsequently centrifuged to remove particulates. Supernatants were stored at −20°C. Primers (Geno-mechanix, Gainesville, FL) from the prior study were used for SYBR green I or TaqMan detection at 100 nM or 900 nM and with 1× SYBR green I dye (Cepheid) or 0.25 mM TaqMan probe (Applied Biosystems), respectively. DNA template (2 μl) and water were added to QPCR reactions for a total volume of 25 μl. The TaqMan protocol consisted of incubation at 50°C for 2 min followed by denaturation at 95°C

* Corresponding author. Mailing address: University of Florida, Department of Food Science and Human Nutrition, P.O. Box 110370, Gainesville, FL 32611. Phone: (352) 392-1991, ext. 311. Fax: (352) 392-9467. E-mail: acw@ufl.edu.

▽ Published ahead of print on 28 September 2007.

for 10 min and 40 cycles of 15 s at 95°C and 60°C for 1 min. The SYBR green I assay used 2 min at 95°C, followed by 40 cycles of the parameters given above.

QPCR examination of DNA from *V. vulnificus* ($n = 25$) and non-*V. vulnificus* ($n = 28$) strains (Table 1) showed both TaqMan detection and SYBR green I detection were 100% sensitive and species specific for *V. vulnificus*. Cycle threshold (C_T) values (number of cycles required to reach threshold for detection) for SYBR green I detection of *V. vulnificus* strains were comparable to those for TaqMan QPCR, with mean C_T values (± standard deviations) of 16.48 ± 0.79 and 16.61 ± 0.87, respectively. All *V. vulnificus* strains were positive by TaqMan assay, while nontarget species were all negative, including false-positive strains (shown in bold in Table 1) described in the prior report (19). Although the SYBR green I assay detected C_T values above threshold for nontarget strains, detection occurred only after extended PCR cycling (mean number of cycles, 34.86 ± 2.28) and is likely to be a consequence of artifactual signal (22). First-derivative analysis of melting curves (melting temperature) provides a sensitive discrimination of nucleotide differences in the DNA sequence of amplicons (21, 22), and species-specific detection of PCR product by SYBR green I was confirmed by single melt peaks with consistent values (mean, 88.02 ± 0.26) from *V. vulnificus* strains (Table 1). In contrast, melt peak values for nontarget species averaged >22 standard deviations apart from the means of positive controls.

The strains examined in this study included other *Vibrio* species that have genes encoding hemolysins that are somewhat related to *vvhA*. Our prior work with the *vvhA* TaqMan probe did not show any cross-reaction with nontarget species; however, false negatives were reported for some nontarget species in another report but only after extensive PCR cycling (19). These false-negative strains were generously provided by A. K. Bej and were included in the present comparison of TaqMan and SYBR green I QPCR detection (shown in bold in Table 1). Although some species (i.e., *Vibrio cholerae*, *V. fluvialis*, and *Aeromonas hydrophila*) exhibit hemolysins with limited deduced amino acid similarity (about 30%) to VvhA, BLAST two-sequence or genomic comparisons did not indicate significant identity to *vvhA* probes and primers at the nucleotide level. The present results also did not show false-negative amplification for any of these strains by TaqMan PCR, and SYBR melt curve analysis confirmed that PCR products detected after extensive cycling (>30 cycles) were not related to a *V. vulnificus* product. Therefore, we conclude that the prior report of a false-negative signal was not based on amplification of homologous DNA.

QPCR-MPN of artificially inoculated PHP oysters. In order to compare assay sensitivities of QPCR-MPN versus standard MPN, PHP oyster homogenates were seeded with known concentrations of *V. vulnificus*. Oysters were obtained immediately postharvest from Leavins Seafood, and the PHP protocol was performed on site in Apalachicola, FL. High initial numbers (>10^4 CFU/g) of *V. vulnificus* bacteria are required for PHP validation (11); therefore, oysters were heat abused prior to processing by overnight incubation (18 to 20 h) at 26°C in order to elevate *V. vulnificus* numbers, followed by refrigeration of oysters at 4°C for 5 to 6 h. Ultralow-temperature PHP oysters were briefly (<30 min) exposed to liquid nitrogen aspersion in

TABLE 1. Specificity and sensitivity of *V. vulnificus* QPCR detection with SYBR green I and TaqMan detection

Strain[a]	QPCR result[b]		
	TaqMan (C_T)	SYBR green I (C_T)	Melt peak (temp [°C])
Target strains			
Vibrio vulnificus			
1009	16.46	16.15	88.29
MO6-24/O	16.14	16.17	88.10
MLT365	ND[c]	18.29	88.15
6353	16.45	15.92	87.91
MLT367	17.21	17.6	88.42
CVD752	15.94	14.87	88.26
345/T	15.60	16.36	87.77
BO6312	17.56	16.44	88.12
5C1326	ND	16.16	88.14
NJMSA	15.91	15.65	87.83
UNCC1015	15.92	15.98	87.98
CVD737	ND	16.13	87.93
LC4	15.62	16.27	87.86
UNCC9	ND	16.32	88.02
85A667	ND	15.61	87.92
1015	16.16	15.87	88.13
345/O	16.56	16.64	87.91
80363	15.72	16.09	88.78
LC4/T	16.95	17.29	88.13
E4125	16.49	15.62	87.83
2400112	18.2	17.34	88.08
52785	ND	17.71	87.46
EDL174	ND	16.5	87.91
MLT403	17.13	17.31	87.77
LL728	17.69	17.29	87.81
Nontarget strains			
***Aeromonas hydrophila* 7965**	0	34.77	70.76
Escherichia coli			
JM109	0	37.42	82.68
HB101	0	35.12	79.43
Listeria monocytogenes	0	36.11	78.15
Pseudomonas aeruginosa	0	35.15	86.4
***Plesiomonas shigelloides* 14029**	0	35.12	76.69
Salmonella enterica serovar Cholerasius 10708	0	36.04	77.97
Salmonella enterica subsp. *enterica* 10112	0	37.64	62.4
Salmonella enterica serovar Enteritidis 13076	0	39.38	63.37
Salmonella enterica serovar Enteritidis 14050	0	38.99	62.66
V. cholerae			
JVY212	0	34.7	79.47
JVB 52	0	33.38	74.24
JVY210	0	28.3	73.88
JVB 25	0	30.36	74.9
2076	0	35.06	79.59
A5	0	35.44	79.59
V. alginolyticus	0	33.18	77.16
V. fischeri ES114	0	38.44	63.17
***V. fluvialis* 1959-2**	0	33.14	78.04
***V. furnissii* 1958-83**	0	34.35	78.76
***V. hollisae* 89A7053**	0	31.37	78.07
V. parahaemolyticus			
LM 5674	0	31.93	72.51
10290	0	34.32	72.71
LM 4892	0	36.31	78.76
N4 3483R	0	39.27	78.9
NY3547	0	33.06	71.91
NVY3483	0	33.06	86.8
TX2103	0	33.14	86.35

[a] Strains in bold were reported to be positive by a prior study (16).

[b] C_T values are shown for QPCR as described in the text, with melt peak analysis results for the SYBR green I assay. The average values for the target strains were a C_T of 16.61 ± 0.87 for the TaqMan assay, a C_T of 16.48 ± 0.79 for the SYBR green I assay, and a melt peak of 88.02°C ± 0.26°C. The average values for the nontarget strains were a C_T of 0 for the TaqMan assay, a C_T of 34.81 ± 2.66 for the SYBR green I assay, and a melt peak of 75.90°C ± 6.75°C.

[c] ND, not done.

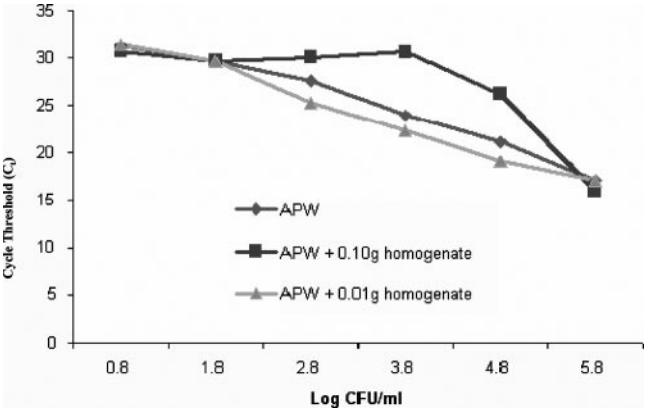

FIG. 1. QPCR standard curve of *V. vulnificus* in APW with or without oyster homogenate. QPCR was performed for dilutions of *V. vulnificus* seeded in triplicate samples of APW with or without addition of oyster homogenate (0.01 g or 0.10 g). C_T values for APW alone or with addition of 0.01 g oyster homogenate showed linearity ($R^2 = 0.99$) at concentrations of 1.8 to 5.8 log CFU/ml. Standard deviations of the means ranged from 0.01 to 0.60.

a freezing tunnel, followed by direct immersion in liquid nitrogen (−87°C) until the meat detached from the shell. Frozen oysters were subsequently kept in extended (≥21 days) dry storage at −20°C in order to obtain *V. vulnificus*-free oysters for seeding studies. PHP oysters ($n = 12$) were homogenized (Warring blender) in PBS (1:2, wt/wt), and dilutions of overnight cultures of *V. vulnificus* M06-24/O in alkaline peptone water (APW) (1 ml) were inoculated into triplicate APW enrichment broths (8 ml) containing either PBS (1 ml) alone or 0.01 or 0.10 g oyster homogenate (1 ml) in PBS. Three-tube replicates of seeded enrichment broth cultures were assessed at 0 and 24 h postincubation at 37°C by SYBR green I or TaqMan QPCR as described above and also by standard MPN, as confirmed by growth of typical colonies isolated on modified colistin polymyxin cellobiose (mCPC) agar (12) incubated at 40°C. The QPCR standard curve for each experiment was based on plate counts of dilutions of *V. vulnificus* on nonselective T1N1 agar (12) as determined immediately postinoculation. Positive (target DNA) and negative (nontarget DNA and no template) controls were included for all QPCR assays. Media were purchased from Fisher Scientific or Difco.

The standard curve for QPCR of *V. vulnificus* in APW with or without the addition of oyster homogenate demonstrated a linear range of detection from about 10^2 to 10^5 for 0.01 g homogenate, with increasing C_T values for decreasing inocula (Fig. 1). However, a significant loss of sensitivity was observed with the addition of 0.10 g oyster tissue at lower inocula. Confirmation of positive samples in seeded homogenates prior to growth in APW was about 100-fold more sensitive by QPCR melt peak than by recovery on mCPC (Table 2). However, after 24 h of enrichment all concentrations of seeded homogenates were positive, as indicated by both growth on mCPC and SYBR QPCR melt peak for both 0.10- and 0.01-g homogenates. SYBR green I and TaqMan QPCR results after 24 h of enrichment were similar, and C_T values ranged from 15.55 to 20.72 or 16.74 to 20.27, respectively (Table 2). Duplicate experiments showed identical MPN results and similar C_T values at 24 h of incubation (not shown). Results confirmed that approximately one cell in the original inoculum could be detected by QPCR-MPN, in agreement with previous reports (1, 18, 19, 20).

QPCR-MPN validation of oyster PHP. Validation of ultralow freezing as a PHP method for oysters was conducted using both standard and QPCR methodologies for confirmation of MPN. SYBR green I detection was selected because results with this dye in the seeding studies described above

TABLE 2. Detection of *V. vulnificus* in artificially inoculated APW enrichment[a]

Inoculum (log CFU/ml)[b]	% Positive samples before APW enrichment		% Positive samples after APW enrichment		Postenrichment C_T by QPCR	
	mCPC	QPCR melt	mCPC	QPCR melt	SYBR green I	TaqMan
0.01 g oyster homogenate						
5.40	100	100	100	100	17.94 ± 0.56	16.74 ± 0.44
4.40	100	100	100	100	18.40 ± 0.40	16.97 ± 0.34
3.40	100	100	100	100	17.90 ± 0.58	17.64 ± 0.20
2.40	33	100	100	100	17.90 ± 0.56	16.83 ± 0.09
1.40	0	100	100	100	17.70 ± 0.60	18.56 ± 0.32
0.40	0	33	100	100	18.70 ± 0.21	17.86 ± 0.30
0.04	0	0	100	100	20.01 ± 2.09	19.87 ± 2.44
Uninoculated	0	0	0	0	35.38 ± 0.25	0.00
0.10 g oyster homogenate						
5.40	100	100	100	100	15.84 ± 0.27	16.99 ± 0.77
4.40	100	100	100	100	16.40 ± 0.07	16.76 ± 0.10
3.40	33	100	100	100	16.29 ± 0.02	17.57 ± 0.21
2.40	0	33	100	100	17.01 ± 1.61	17.44 ± 1.41
1.40	0	0	100	100	17.93 ± 2.89	18.35 ± 1.63
0.40	0	0	100	100	15.55 ± 0.70	16.92 ± 0.23
0.04	0	0	100	100	20.72 ± 1.27	20.27 ± 1.43
Uninoculated	0	0	0	0	33.18 ± 2.43	0.00

[a] Detection of *V. vulnificus* in APW, determined as percent positive samples from three tubes, was based either on observation of *V. vulnificus* typical colonies on selective agar (mCPC) or on melt peak analysis (QPCR) for pre- and postenrichment. Values are the means (±standard deviations) of duplicate experiments with identical results.

[b] *V. vulnificus* inocula (log CFU/ml as determined by plate count) for seeding of MPN enrichment of PHP oyster homogenates (0.01 or 0.10 g).

TABLE 3. Comparison of standard MPN to QPCR-MPN analysis of PHP oyster samples

Oyster lot	Treatment[a]	Avg (±SD) log MPN/g[b]	
		FDA MPN	QPCR MPN
1	Pre-PHP	2.7 ± 1.5	3.2 ± 0.3
2	Pre-PHP	4.4 ± 0.4	4.8 ± 0.2
3	Pre-PHP	4.1 ± 1.0	4.3 ± 0.5
1	PHP 1D	0.9 ± 0.5	1.7 ± 1.1
2	PHP 1D	1.9 ± 0.6	2.3 ± 0.3
3	PHP 1D	3.7 ± 0.3	3.8 ± 0.2
1	PHP 21D	1.5 ± 0.4	2.0 ± 0.1
2	PHP 21D	0.6 ± 0.3	0.6 ± 0.3
3	PHP 21D	0.5 ± 0.0	0.5 ± 0.0
4	PHP 21D	1.1 ± 0.2	0.9 ± 0.3

[a] Individual oyster lots ($n = 4$) were heat abused by incubation at 26°C for 24 h (pre-PHP), followed by processing with ultralow freezing in liquid nitrogen and frozen storage at −10°C for 1 (PHP 1D) or 21 (PHP 21D) days following PHP.
[b] For each lot, oysters ($n = 12$) were sampled in triplicate, and log MPN/g values were determined by the standard FDA bacteriological analytical manual method (FDA MPN) or by MPN using QCPR confirmation with SYBR green I (QPCR-MPN), as described in the text. Lots 1 to 3 were examined before and after PHP, and lot 4 was examined only at 21 days after PHP.

were comparable to results with TaqMan but with lower cost and additional confirmation by melt peak analysis. A comparison of QPCR-based MPN to standard MPN used concurrent examinations of four independent lots ($n = 3$ samples/lot) of oysters ($n = 12$ for each sample) that were processed by ultralow freezing as described above. Lots 1 to 3 were evaluated by MPN prior to processing and at days 1 and 21 of frozen storage. An additional lot (lot 4) was evaluated only on day 21. Homogenized oysters were prepared as described above and serially diluted in PBS to yield 1.0 to 0.00001 g for three-tube enrichment cultures (10 ml). *V. vulnificus*-positive growth was determined by standard MPN on mCPC as described above but with DNA probe confirmation (26) and by SYBR green I QPCR analysis. Positive (target DNA) and negative (nontarget DNA and no template) controls along with standard curve analysis of extracted DNA from dilutions of *V. vulnificus* cells in APW were included with each sample for quality control.

QPCR-MPN values for all lots were comparable ($R^2 = 0.97$ by Pearson's correlation coefficient) to standard MPN results for confirmation of growth of *V. vulnificus* following enrichment (Table 3). A total of 1,232 enrichment culture tubes were examined, with 84.9% agreement between both assays: 14.8% of cultures were QPCR positive and probe negative, and only one sample (0.3%) was probe positive and PCR negative. Discrepancies between the two assays were observed only at higher homogenate concentrations (1.0 to 0.1 g enrichment culture), while results with more diluted concentrations (0.01 to 0.0001 g) were consistent for both assays. These results agree with prior observations that MPN values derived from higher concentrations of homogenate plated on CPC may yield false-negative results that are inconsistent with positive results from more dilute concentrations of homogenate (12). Thus, high concentrations of homogenates appear to inhibit either growth of *V. vulnificus* in APW or recovery on mCPC. In our study, discrepant samples showed positive melt peaks but with high C_T values (C_T of >30), indicating very little growth in APW.

Both dead cells and viable but not culturable cells are detected by PCR (3) and represent a possible source of contamination for PCR-based MPN. However, the standard MPN assay is dependent on viability of target bacteria, and agreement of QPCR-MPN with this assay demonstrated that residual DNA from nonviable or nonculturable cells did not contribute significantly to QPCR-MPN. Enriched samples that were PCR positive but negative on selective media invariably were derived from higher concentrations of homogenates that were falsely negative on mCPC, as indicated by agreement of positive mCPC and QPCR results in more-diluted inocula of the same sample. DNA from dead cells is likely to be degraded after overnight incubation of oyster homogenates or diluted below the limit of QPCR detection. For example, the addition of 0.10 or 0.01 g of oyster tissue containing 10^4 MPN/g of *V. vulnificus* would yield about 10^1 to 10^2 CFU/ml, respectively, in 10 ml of APW enrichment. Thus, without bacterial growth, the level of *V. vulnificus* DNA in broth culture is at or below the limit of detection for QPCR. Bacterial growth during MPN enrichment amplifies the signal for QPCR by increasing cell density and results in C_T values equivalent to $>10^4$ to 10^5 CFU/ml based on seeding studies, greatly exceeding possible contamination DNA from dead cells. Additional DNA purification or concentration steps may increase QPCR assay sensitivity but could also increase false-positive detection of DNA from dead cells.

Summary. These data demonstrated that QPCR provides a sensitive and cost-effective alternative to standard methods for confirmation of MPN. Field trials indicated that QPCR offered an improved confirmatory assay compared to the standard method, as QPCR showed more-sensitive detection at higher concentrations of oyster tissue. Other studies have also reported a lack of growth of some strains of *V. vulnificus* (serovar E) on CPC or growth of nontarget species (15). Results support adoption of a QPCR confirmation to ensure detection of *V. vulnificus* at higher concentrations of oyster homogenate. Our data conflicted with a prior report (19) of detection of false positives by the TaqMan assay. Detection of non-*Vibrio* species (*Aeromonas* and *Pseudomonas* species) cannot be attributed to primer hybridization with homologous DNA, as these species do not have sequences with sufficient DNA identity to align with *V. vulnificus* primers by two-sequence BLAST analysis. We found that discrimination of low-level detection (C_T values that were just slightly above threshold after >30 cycles of extended PCR cycling) was provided by the melt peak analysis in the present study. False-positive data may result from numerous differences in methodology (extraction protocol, template concentration, reagents, equipment, etc.) or may reflect contamination, PCR artifact, or excess template. Development of robust, highly reproducible QPCR assays requires optimization, standardization, and interlaboratory verifications that are difficult to achieve but are needed for adoption of these protocols by the industry. The application of internal control standards, as opposed to the external controls and standard curve analysis used herein, would also simplify and enhance this assay. Results show that combining QPCR with MPN increased assay reliability and sensitivity compared

to standard methods and support the application of this technology for PHP validation of oysters.

This research was funded in part by Sea grants R/LR-Q-26A and R/LF-Q-30 and a USDA special grant.

Technical assistance was provided by Joel Fernandes, Koo-Whang Chung, Charlene Burke, and ABC Research, Inc. Oysters were provided by Leavins Seafood.

REFERENCES

1. **Blackstone, G. M., J. L. Nordstrom, M. C. L. Vickery, M. D. Bowen, R. F. Meyer, and A. Depaola.** 2003. Detection of pathogenic *Vibrio parahaemolyticus* in oyster enrichments by real time PCR. J. Microbiol. Methods **53:**149–155.
2. **Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heulein.** 1979. Disease caused by a marine vibrio: clinical characteristics and epidemiology. N. Engl. J. Med. **300:**1–5.
3. **Brauns, L. A., M. C. Hudson, and J. D. Oliver.** 1991. Use of the polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. Appl. Environ. Microbiol. **57:**2641–2655.
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. **Cook, D. W., and A. D. Ruple.** 1992. Cold storage and mild heat treatment as processing aids to reduce the numbers of *Vibrio vulnificus* in raw oysters. J. Food. Prot. **55:**985–989.
6. **Dalsgaard, A., N. Frimodt-Moller, B. Bruun, L. Hoi, and J. L. Larsen.** 1996. Clinical manifestations and molecular epidemiology of *Vibrio vulnificus* infections in Denmark. Eur. J. Clin. Microbiol. Infect. Dis. **15:**227–232.
7. **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.
8. **Feldhusen, F.** 2000. The role of seafood in bacterial foodborne diseases. Microbes Infect. **2:**1651–1660.
9. **Gulig, P. A., K. L. Bourdage, and A. M. Starks.** 2005. Molecular pathogenesis of *Vibrio vulnificus*. J. Microbiol. **43:**118–131.
10. **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.
11. **Interstate Shellfish Sanitation Conference.** 2003. Issue relating to a *Vibrio vulnificus* risk management plan for oysters. ISSC, Columbia, SC.
12. **Kaysner, C. A., and A. DePaola.** 2004. *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and other *Vibrio* spp. Bacteriological analytical manual online, 8th ed. U.S. FDA, Center for Food Safety and Applied Nutrition. http://www.cfsan.fda.gov/~ebam/bam-toc.html.
13. **Kelly, M. T.** 1982. Effect of temperature and salinity on *Vibrio* (*Beneckea*) *vulnificus* occurrence in a Gulf Coast environment. Appl. Environ. Microbiol. **44:**820–824.
14. **Linkous, D. A., and J. D. Oliver.** 1999. Pathogenesis of *Vibrio vulnificus*. FEMS Microbiol. Lett. **174:**207–214.
15. **Macián, M. C., C. R. Arias, R. Aznar, E. Garay, and J. J. Pujalte.** 2000. Identification of *Vibrio* spp. (other than *V. vulnificus*) recovered from CPC agar from marine natural samples. Int. Microbiol. **3:**51–53.
16. **Motes, M. L., A. DePaola, D. W. Cook, J. E. Veazey, J. C. Hunsucker, W. E. Garthright, R. J. Blodgett, and S. J. Chirtel.** 1998. Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). Appl. Environ. Microbiol. **64:**1459–1465.
17. **Oliver, J. D., R. A. Warner, and D. R. Cleland.** 1983. Distribution of *Vibrio vulnificus* and other lactose-fermenting vibrios in the marine environment. Appl. Environ. Microbiol. **45:**985–998.
18. **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.
19. **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.
20. **Panicker, G., M. L. Myers, and A. K. Bej.** 2004. Multiplex PCR detection of clinical and environmental strains of *Vibrio vulnificus* in shellfish. Can. J. Microbiol. **50:**911–922.
21. **Randa, M. A., M. F. Polz, and E. Lim.** 2004. Effects of temperature and salinity on *Vibrio vulnificus* population dynamics as assessed by quantitative PCR. Appl. Environ. Microbiol. **70:**5469–5476.
22. **Ririe, K. M., R. P. Rasmussen, and C. T. Wittwer.** 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem. **245:**154–160.
23. **Strom, M. S., and R. N. Paranjpye.** 2000. Epidemiology and pathogenesis of *Vibrio vulnificus*. Microbes Infect. **2:**177–188.
24. **Tamplin, M. L., G. E. Rodrick, N. J. Blake, and T. Cuba.** 1982. Isolation and identification of *Vibrio vulnificus* from two Florida estuaries. Appl. Environ. Microbiol. **44:**1466–1470.
25. **Wright, A. C., R. T. Hill, J. A. Johnson, M. C. Roghman, R. R. Colwell, and J. G. Morris, Jr.** 1996. Distribution of *Vibrio vulnificus* in the Chesapeake Bay estuaries. Appl. Environ. Microbiol. **62:**717–724.
26. **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.