Real-Time PCR Quantification of *Vibrio parahaemolyticus* in Oysters Using an Alternative Matrix


1Department of Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294-1170; 2U.S. Food and Drug Administration, Gulf Coast Seafood Laboratory, P.O. Box 158, Dauphin Island, Alabama 36528-0158; 3U.S. Food and Drug Administration, Office of Math and Statistics, 5100 Paint Branch Parkway, College Park, Maryland 20740; and 4Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, Georgia 30333, USA

MS 04-128: Received 30 March 2004/Accepted 9 June 004

**ABSTRACT**

This study examined the relationship between levels of total *Vibrio parahaemolyticus* found in oyster tissues and mantle fluid with the goal of using mantle fluid as a template matrix in a new quantitative real-time PCR assay targeting the thermostable direct hemolysin gene (*tlh*) for the enumeration of total *V. parahaemolyticus* in oysters. Oysters were collected near Mobile Bay, Ala., in June, July, and September and tested immediately after collection and storage at 26°C for 24 h. Initial experiments using colony hybridization targeting *tlh* demonstrated that natural *V. parahaemolyticus* levels in the mantle fluid of individual oysters were strongly correlated (*r* = 0.85, *P* < 0.05) with the levels found in their tissues. When known quantities of cultured *V. parahaemolyticus* cells were added to real-time PCR reactions that contained mantle fluid and oyster tissue matrices separately pooled from multiple oysters, a strong linear correlation was observed between the real-time PCR cycle threshold and the log concentration of cells inoculated into each PCR reaction (mantle fluid: *r* = 0.98, *P* < 0.05; and oyster: *r* = 0.99, *P* < 0.05). However, the mantle fluid exhibited less inhibition of the PCR amplification than the homogenized oyster tissue. Analysis of natural *V. parahaemolyticus* populations in mantle fluids using both colony hybridization and real-time PCR demonstrated a significant (*P* < 0.05) but reduced correlation (*r* = −0.48) between the two methods. Reductions in the efficiency of the real-time PCR that resulted from low population densities of *V. parahaemolyticus* and PCR inhibitors present in the mantle fluid of some oysters (with significant oyster-to-oyster variation) contributed to the reduction in correlation between the methods that was observed when testing natural *V. parahaemolyticus* populations. The *V. parahaemolyticus*–specific real-time PCR assay used for this study could estimate elevated *V. parahaemolyticus* levels in oyster mantle fluid within 1 h from sampling time.

*Vibrio parahaemolyticus* is a gram-negative marine bacterium found in estuaries worldwide, but it is especially abundant in warm, brackish waters such as those around the Gulf of Mexico (2, 10, 12, 15, 17, 19). Infection from *V. parahaemolyticus* is the leading cause of reported cases of bacterial gastroenteritis associated with seafood consumption in the United States (1, 22). Risk factors include ingestion of raw or undercooked seafood, improper storage of seafood, and poor handling of seafood during preparation (9). Raw molluscan shellfish are of particular concern, because they concentrate this pathogen from surrounding waters during filter feeding and are often consumed raw. A 200-fold higher distribution of this pathogen in shellfish than in the surrounding waters has been reported (10).

From 1981 to 1997, cases of *V. parahaemolyticus* were sporadic (9); however, during 1997 to 1998 there were four outbreaks associated with consumption of raw oysters in Washington (5), Texas (8), and New York (6), where more than 700 cases were reported. The outbreaks in Texas and New York were primarily associated with the pandemic serotype O3:K6 (6, 8).

These outbreaks focused attention toward the monitoring of *V. parahaemolyticus* levels (7, 12) and on research of the growth and survival of this organism in shellfish (16). These studies have been facilitated by the use of colony hybridization procedures with nonradioactive DNA probes targeting the thermostable hemolysin gene (*tlh*) and the thermostable direct hemolysin gene (*tdh*) for the enumeration of total and pathogenic *V. parahaemolyticus*, respectively (20, 21). In these methods, oyster homogenate is spread plated to nonselective media and incubated overnight, and colony hybridization is performed the following day. These methods have been adopted by the American Public Health Association (18). However, high levels of background microflora in oysters can sometimes produce confluent growth on the plating media, often leading to masking of signal (7). Although conventional PCR methods have been used to detect *V. parahaemolyticus* in oysters with high levels of background microflora (3), they lack quantitative capability unless they are used in conjunction with a most-probable-number (MPN) analysis. However, this serial dilution procedure for obtaining MPN estimates is resource intensive and relatively imprecise compared with colony hybridization (14).

Real-time PCR fluorogenic probes can be used to mea-
sure the quantity of PCR product as it accumulates during each PCR cycle. Using appropriate standards, real-time PCR can be used for direct quantification based on a linear relationship that exists between the real-time PCR cycle threshold (Ct) and the log of the initial target copy number. The Ct is the fractional PCR cycle at which the fluorescent signal significantly rises above background or a defined threshold. A standard curve is generated by plotting the log of the initial target copy number for a set of standards versus the Ct values obtained during real-time PCR. Cycle threshold values for unknowns are then compared with this standard curve to determine the initial target number in a given reaction.

Homogenized oyster tissue is known to contain substances that can severely interfere with PCR (11). It would be desirable to identify a less inhibitory component of the oyster matrix that contains target organisms in numbers indicative of what may be found in an oyster tissue homogenate as a whole. This component could then be used as a representative sample matrix for direct PCR analysis of oysters.

Oyster mantle fluid (the liquid inside the shell that bathes the oyster) and hemolymph have been examined and compared with oyster tissue in regard to levels of *Vibrio vulnificus* (13, 22), but *V. parahaemolyticus* levels have not been previously examined in these matrices (23). Preliminary experiments with hemolymph indicated much lower *V. parahaemolyticus* densities than were found in mantle fluid or oyster tissues and demonstrated that hemolymph is also severely inhibitory to PCR (unpublished data). In the present study, we developed a real-time PCR assay targeting the *tlh* gene for the enumeration of total *V. parahaemolyticus* and examined whether mantle fluid might be suitable as an alternative matrix for the determination of *V. parahaemolyticus* levels in oysters using both colony hybridization with DNA probe and real-time PCR.

**MATERIALS AND METHODS**

**Bacterial strains.** A clinical O3:K6 serotype *V. parahaemolyticus* strain associated with the 1998 outbreak in Texas was used to inoculate mantle fluid and oyster tissues for the purpose of producing standard curves. Specificity of the real-time PCR assay for *tlh* was determined using *V. parahaemolyticus* strains from various sources in the United States and abroad and with other bacterial species, including closely related vibrios from our culture collection (Table 1).

**Sample collection.** Twenty Gulf Coast oysters (*Crassostrea virginica*) were collected within a 1-m radius at a depth of approximately 1 m from Dauphin Island Bay located near Mobile Bay, Ala., in June, July, and September 2001. The water temperature at the sampling site was measured using a mercury thermometer, and the salinity of the water was determined with a refractometer (Reichert-Jung, Cambridge Instruments, Buffalo, N.Y.). All oysters were scrubbed with a brush under running tap water and analyzed individually. For each oyster, an oyster knife was inserted (<1.0 cm) between the shells on the bill end of the oyster and twisted slightly, being careful not to cut any of the tissues, and all of the mantle fluid (range, 0.64 to 10.20 g) was drained into a sterile petri dish. For each oyster, a few drops of mantle fluid were used for refractive index measurement for comparison to the refractive index of the overlying sea water, since a higher reading in the mantle fluid than in the harvest waters might be indicative of contamination with hemolymph from lacerated tissues. Bacteriological analyses were conducted immediately on a portion of the mantle fluid, and an aliquot was aseptically transferred to a 1.5-ml microcentrifuge tube and stored either on ice or at 4°C before PCR analysis, which was conducted within 24 h of sample collection. After the mantle fluid was removed, the oysters were shocked, weighed (range, 12.98 to 51.37 g), and placed into blenders. Ten oysters were placed in a 3°C cooler immediately after collection and analyzed individually on the same day of collection for *V. parahaemolyticus* levels using the methods described below. The remaining 10 oysters were immediately incubated at 26°C in plastic bags and tested individually after storage for 24 h.

**Bacteriological analysis.** Each oyster was individually homogenized with an equal weight (1:1) of alkaline-peptone water (APW; 10 g/liter of peptone, 10 g/liter of NaCl, pH 8.5) for 45 s using a sterile Eberbach blender container (250-ml capacity) and a Waring blender base. T<sub>2</sub>N<sub>4</sub> agar (1% tryptone, 3% NaCl, 2% agar, pH 7.2) plates were spread plated with 0.1 g (0.2 g of 1:1 APW-oyster homogenate) and 0.01 g (100 μl of 1:10 APW-oyster dilution) portions of the homogenized oyster tissue. The plates were incubated overnight at 35°C, and colony lifts followed by colony hybridization with DNA probes were performed as previously described (20). Briefly, colony lifts were prepared on Whatman 541 filters; total and pathogenic *V. parahaemolyticus* were identified using alkaline phosphatase–labeled DNA probes for *tlh* (DNA Technology, Aarhus, Denmark) (20, 21). Portions of mantle fluid (0.1 and 0.01 ml) were also analyzed by spread plating or colony lift and hybridization with DNA probe as described above.

**Real-time PCR.** Primer Express software from Applied Biosystems (Foster City, Calif.) was used to design oligonucleotide primers and a fluorogenic probe targeting a conserved species-specific, 62-bp region of the *V. parahaemolyticus* *tlh* gene (GenBank Accession no. M36437) (24). The forward primer 5'-

<table>
<thead>
<tr>
<th>TABLE 1. Bacterial species tested for specificity with the <em>tlh</em> real-time PCR assay&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td><em>Listeria innocua</em></td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
</tr>
<tr>
<td><em>L. welshii</em></td>
</tr>
<tr>
<td><em>L. seeligeri</em></td>
</tr>
<tr>
<td><em>Salmonella</em></td>
</tr>
<tr>
<td><em>Vibrio alginolyticus</em></td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
</tr>
<tr>
<td><em>V. fluvialis</em></td>
</tr>
<tr>
<td><em>V. hollisae</em></td>
</tr>
<tr>
<td><em>V. metchnikovii</em></td>
</tr>
<tr>
<td><em>V. mimicus</em></td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
</tr>
</tbody>
</table>

<sup>a</sup>A total of 16 species and 89 strains were tested; all of the strains were correctly identified (89 of 89).

---

J. Food Prot., Vol. 67, No. 11 REAL-TIME PCR QUANTIFICATION OF *V. PARAHAEOMLYTICUS* 2425
TABLE 2. Means and standard deviations of total *V. parahaemolyticus* (log/g)

<table>
<thead>
<tr>
<th>Month</th>
<th>Oyster</th>
<th>Mantle</th>
<th>Oyster</th>
<th>Mantle</th>
</tr>
</thead>
<tbody>
<tr>
<td>June</td>
<td>2.90 A1a (0.91)</td>
<td>3.35 A1 (0.45)</td>
<td>4.04 A2 (0.50)</td>
<td>4.13 A2 (0.50)</td>
</tr>
<tr>
<td>July</td>
<td>2.88 A1 (0.36)</td>
<td>2.66 B1 (0.35)</td>
<td>4.24 A2 (0.39)</td>
<td>4.27 A2 (0.44)</td>
</tr>
<tr>
<td>September</td>
<td>2.47 A1 (0.26)</td>
<td>2.35 B1 (0.42)</td>
<td>3.52 A2 (0.45)</td>
<td>3.55 A2 (0.59)</td>
</tr>
</tbody>
</table>

*a* Mean log densities marked with the same letter within a column are not significantly different (*P* < 0.05) from each other. Mean log densities marked with the same numeral within a row are not significantly different (*P* < 0.05) from each other. Standard deviations of log densities are shown in parentheses.

AACTTCTGCGCGCGAAGAG-3’ and the reverse primer 5'-CGGTTGATGTCACAAAGGA-3’ were synthesized by Invitrogen (Carlsbad, Calif.). The fluorgenic probe 5’-FAM-CCGCTGCCTACGAAACCG-QSY7-3’ was synthesized and provided by the Centers for Disease Control Core Synthesis Facility (Atlanta, Ga.). FAM represents the carboxyfluorescein reporter and QSY-7 represents the dark quencher molecule. Real-time PCR reactions were conducted in a 25-µl volume using 1× Platinum Taq PCR Buffer with 7 mM MgCl2 (Invitrogen), 0.2 µM dNTPs (Invitrogen), 0.3 µM of each primer, 0.05 µM fluorescent probe, and 1.25 U Platinum Taq DNA polymerase (Invitrogen). PCR amplification was conducted using the following cycling parameters: an initial denaturation at 94°C for 120 s followed by 50 cycles of amplification, with each cycle consisting of denaturation at 94°C for 15 s and primer annealing and extension at 60°C for 30 s. For each sample, either 2.5 µl of mantle fluid or 2.5 µl of oyster tissue homogenate (50% oyster tissue and 50% APW) was used as template for amplification. Real-time PCR amplification was run on a Smart Cycler instrument (Cepheid, Sunnyvale, Calif.).

The Smart Cycler instrument software was set to take fluorescence readings at the end of each annealing-extension step, and a sample was considered to be positive at the fractional PCR cycle at which the fluorescence value was observed to rise above a threshold of 30 fluorescence units above baseline fluorescence (Ct). Amplified products (10 µl of each sample tested) were examined by gel electrophoresis to confirm that the results reported by the Smart Cycler correlated with amplification of a product of the expected size. The PCR products were separated in 5% (wt/vol) Metaphor agarose and EtBr gel (FMC Bioproducts, Rockland, Maine) in TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.3) electrophoretic buffer. Gels were photographed using an Alpha Imager2000 documentation and analysis system (Alpha Innotech Corporation, San Leandro, Calif.).

**Standard curves.** Standard curves were generated (in duplicate) during each monthly experiment using sterile MilliQ water, mantle fluid, and homogenized oyster tissue from the same oyster as matrices, which were inoculated with *V. parahaemolyticus* strain TX-2103 as a control. The inoculum levels were adjusted for indigenous *V. parahaemolyticus* in the mantle fluid and oyster tissues as determined by colony hybridization. The culture of TX-2103 used for inoculation was grown for 6 h at 35°C in APW (pH 8.5) and serially diluted in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.3), and 100 µl of selected dilutions was plated in triplicate on TlN4 agar. From each serial dilution of cells prepared above, crude DNA template was prepared by boiling a 1 ml aliquot for 10 min to lyse the cells and inactivate nucleases (4). The plates were incubated overnight at 35°C and the number of CFUs was determined. For each matrix above, real-time PCR targeting the *tlh* gene was performed using a mixture of 2.5 µl of matrix with either 1 or 2 µl of boiled cells (at each dilution) to generate standard curves in each matrix by plotting the log CFU/ml (based on the plate counts and dilutions) versus Ct.

**Statistical analysis.** Differences in bacterial concentrations in oysters versus mantle fluid at harvest, after incubation, and across sample collection months were analyzed by regression, analysis of variance, and Pearson correlation methods. The relationship between PCR Ct and bacterial concentration was determined in the same manner as for standard curves, with differences in correlation assessed by Fisher’s z transform method. All bacterial concentrations determined by colony hybridization were converted to log units for statistical analysis. Only 1 of 60 samples enumerated by colony counts was undetectable for *V. parahaemolyticus* and was assigned a value of half the limit of detection (5 CFU/ml) based on the largest sample volume examined. With respect to PCR detection of natural populations of *V. parahaemolyticus* in mantle fluid, 23 of the 60 samples did not yield any positive signal and an additional three samples had a Ct above 44. These outcomes were considered undetectable and were excluded from statistical analysis. Calculations were performed using Excel 2000 (Microsoft, Seattle, Wash.) and Statistical Analysis System software (SAS Institute, Cary, N.C.). An α level of 0.05 was considered the minimum level for significance.

**RESULTS**

**Real-time PCR targeting the *tlh* gene.** The *tlh* assay correctly identified all *V. parahaemolyticus* tested from diverse sources but none of the near neighbor *Vibrio* spp. or other bacterial species examined (Table 1). It also provided excellent amplification characteristics over a range (4 to 7 mM) of MgCl2 concentrations. Analysis of standard curves generated using serial dilutions of boiled cells showed an excellent linear relationship (correlation coefficient $r^2 = 0.99$, $P < 0.05$) between the log CFU numbers and Ct values across at least six orders of magnitude down to a single cell per reaction (data not shown).

**Levels of *V. parahaemolyticus* in mantle fluid and oyster tissue.** Based on the results of colony hybridization with the *tlh* DNA probe, mean log densities in mantle fluid correlated well with mean densities in oyster tissue (Table 2). For samples collected in the same month and assayed at the same time after harvest, there were no significant differences in the mean level for mantle fluid compared with that of oyster tissue. The mean log levels were significantly higher at 24 versus 0 h for both oyster and mantle fluid. The overall correlation between densities in mantle...
FIGURE 1. Correlation of *V. parahaemolyticus* densities (log CFU per gram) estimated by DNA probe in the mantle fluid versus the oyster tissue of individual oysters harvested during the months of June, July, and September. The triangles represent oysters processed immediately after harvest, and the diamonds represent oysters processed after incubation at 26°C for 24 h.

Fluid versus oyster tissue (Fig. 1) was 0.85 (*P* < 0.05). The correlation was higher after incubation at 26°C (*r* = 0.80, *P* < 0.05) compared with that at time of harvest (*r* = 0.59, *P* < 0.05), but the difference was not statistically significant.

The relationship between real-time PCR Ct values and CFU per gram of *V. parahaemolyticus* was determined by inoculating serial 10-fold dilutions of *V. parahaemolyticus* into sterile MilliQ water, mantle fluid, or oyster homogenate (50% oyster tissue and 50% APW). Gel electrophoresis confirmed that the fluorescence signal correlated with amplified product of the correct size (data not shown). Total *V. parahaemolyticus* levels in the mantle fluids and the oyster homogenates tested were determined by DNA colony hybridization using an alkaline phosphatase–labeled probe for the *tlh* gene. A good linear correlation was observed between the real-time PCR Ct and the log concentration of cells inoculated into each PCR reaction (water: *r*² = 0.99; mantle fluid: *r*² = 0.98; oyster: *r*² = 0.99), with mantle fluid exhibiting less inhibition of the PCR than the homogenized oyster tissue based on Ct (Fig. 2). The amount of inhibition shown by the mantle fluid and oyster tissue based on Ct varied between samples, but throughout the experiments mantle fluid was consistently less inhibitory than the oyster tissue homogenate. For example, in June the Ct values were 3.22, 2.66, and 1.53 greater in mantle fluid than in water when the *V. parahaemolyticus* concentrations were approximately 2, 3, and 4 log CFU/ml for each reaction, respectively. Using oyster tissue homogenate from a single oyster collected in June, corresponding to the mantle fluid collected both at harvest and after 24-h incubation, there was no evidence of PCR inhibition.

Natural populations of *V. parahaemolyticus* in oyster mantle fluid at harvest and populations held at 26°C for 24 h were enumerated by colony hybridization and compared with real-time PCR Cts (Fig. 3). Considering only those samples that were positive for *V. parahaemolyticus* by PCR, the real-time PCR Ct and *V. parahaemolyticus* density determined by colony count were inversely correlated with an overall correlation of *r* = −0.48 (*P* < 0.05). However, there was considerable variation between Cts for samples at comparable densities. Considering only samples at harvest, the correlation was lower and not significant (*r* = −0.38, *P* = 0.20). With respect to samples incubated at 26°C for 24 h, there was less variation at comparable densities and the correlation between Cts and *V. parahaemolyticus* densities was stronger (*r* = −0.59, *P* < 0.05). The largest number of *V. parahaemolyticus* in a mantle fluid sample not detected by real-time PCR was 4.77 log CFU/ml (147 cells per PCR reaction) as estimated by colony hybridization. There was no evidence of PCR inhibition.

FIGURE 2. Standard curves from real-time PCR generated by inoculating known concentrations of *V. parahaemolyticus* into water, mantle fluid, or oyster tissue.

FIGURE 3. Cycle thresholds from real-time PCR of the natural population of *V. parahaemolyticus* in the mantle fluid collected at time of harvest (open circles) or after incubation at 26°C (closed circles). The dashed line is the regression fit of cycle threshold versus *V. parahaemolyticus* density determined by DNA probe for mantle fluid collected both at harvest and after 24-h incubation.
from hemolymph contamination of mantle fluid in any of the samples tested, because the refractometer readings of the mantle fluids were always within 2 ppt of those observed in the harvest waters.

**DISCUSSION**

This study demonstrates that V. parahaemolyticus levels in the mantle fluids of the American oysters (C. virginica) are indicative of the levels in the whole oyster both at harvest and after 26°C storage. Bacteriological analysis by culture or PCR is much simpler with mantle fluid than with oyster tissue, because mantle fluid can be collected and manipulated entirely by pipetting. When oysters are homogenized in a blender, air is introduced, preventing accurate volumetric transfer and necessitating gravimetric determination. Oyster tissues also complicate many common laboratory manipulations, such as pipetting, filtration, and centrifugation. Our results also show that oyster tissues are more inhibitory to real-time PCR detection of V. parahaemolyticus than mantle fluid.

The levels of V. vulnificus in various tissues and fluids from oysters have been studied (13, 23), but similar studies have not been performed for V. parahaemolyticus. In these previous studies with V. vulnificus, cell levels were generally found to be 1 or 2 logs lower in mantle fluid and hemolymph than in oyster tissues (13, 22). In the present study, preliminary experiments with a few oysters indicated that V. parahaemolyticus levels (as determined by colony hybridization with a DNA probe for tll) were more than 2 logs lower in the hemolymph than in the mantle fluid at harvest and as much as 4 logs lower after 24 h storage at 26°C (data not shown). These findings suggest that V. vulnificus and V. parahaemolyticus differ considerably in their ecological relationship with oysters. The association of V. vulnificus with various oyster tissues and hemolymph has been hypothesized to be an important factor in the failure of depuration to reduce V. vulnificus levels in oysters (23). Further study of V. parahaemolyticus levels in various oyster tissues may provide insight into the potential effectiveness of depuration for eliminating V. parahaemolyticus from oysters

The standard curves generated by using real-time PCR with the mantle fluid and oyster tissue inoculated with V. parahaemolyticus demonstrated excellent correlation with colony hybridization results, suggesting that real-time PCR could be used to quantify V. parahaemolyticus in either matrix. However, there was considerably less inhibition of PCR with mantle fluid than with oyster tissue. In fact, in the present study, 2.5 μl of mantle fluid was used as template for PCR, whereas only 1.25 μl of oyster tissue was effectively used per reaction, since the oyster homogenate was diluted 1:1 with APW. The inhibition from oyster tissue would have been much greater (or caused complete inhibition) had 2.5 μl of oyster tissue been used in the reactions. In some samples, the detection sensitivity was 1 to 2 logs greater in the mantle fluid compared with the oyster tissue from the same oyster. Although inhibition of PCR by mantle fluid and oyster tissue varied between the oysters tested, overall the mantle fluid was consistently less inhibitory to real-time PCR than the oyster tissue. The oyster-to-oyster variation of PCR inhibitors in oyster tissue and the low concentration of V. parahaemolyticus in some samples may account for the relatively weak correlation between quantitation by real-time PCR and quantitation by DNA colony hybridization (Fig. 3).

The small volume (2.5 μl) of mantle fluid used as template often contained few or no V. parahaemolyticus cells, especially for samples analyzed immediately after harvest, and this also may have affected the correlation. The limit of detection by PCR in mantle fluid corresponded to a density of 2.11 log CFU/ml, as determined by plate counts. At this density, the expected number of cells is less than 1 per 2.5-μl PCR reaction volume. The quantitative accuracy of real-time PCR can be adversely affected by low target copy number if amplification is even slightly inhibited during the initial cycles of the PCR reaction. This may explain the higher correlation between the Ct and colony hybridization results in mantle fluid of oysters stored at 26°C, which had higher densities of V. parahaemolyticus than those analyzed immediately after harvest. When V. parahaemolyticus densities exceeded 10^4 CFU/g, there appeared to be a much stronger correlation with the Ct.

Based on our initial results, mantle fluid could be an effective matrix for determining the levels of V. parahaemolyticus in oysters. It remains to be examined whether V. parahaemolyticus levels in mantle fluid correspond to levels in oyster tissue for other oyster species or for C. virginica oysters harvested from waters other than the Gulf of Mexico. The environmental and ecological considerations that affect the relationship between bacterial levels in mantle fluid and oyster tissues needs to be further examined.

DNA purification techniques can be used to improve detection from mantle fluid in natural samples. In addition, by scaling the PCR reaction volume up to 50 or 100 μl, a greater sample volume could be tested, allowing an increase in the sensitivity of the assay. In a recent study, we also determined that boiling mantle fluid and oyster tissue homogenates before testing by PCR will often significantly reduce PCR inhibition, especially for oyster tissue (unpublished data). Serial samples of mantle fluid can also be collected from a single oyster, which could allow for a better understanding of the growth of this pathogen under different handling conditions such as temperature abuse.

In conclusion, we found that the levels of V. parahaemolyticus in mantle fluid correlate well with the levels in oyster tissue in Gulf Coast oysters. With the development of simple mantle fluid bacterial cell concentration and DNA purification procedures, levels of V. parahaemolyticus in the mantle fluid could be rapidly and accurately determined using real-time PCR. We believe this to be the first application of real-time PCR to quantitate a naturally occurring pathogen in a seafood matrix.

**ACKNOWLEDGMENTS**

This study was supported by the Mississippi Alabama Sea Grant Consortium and the University of Alabama at Birmingham on a research grant (R/SP-1 and NA86RG0039-4) and was also supported by the Sum-
mer Student Internship Program of the U.S. Food and Drug Administration, Washington, D.C.

REFERENCES


