Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Microbiological Methods 53 (2003) 149–155
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

Detection of pathogenic *Vibrio parahaemolyticus* in oyster enrichments by real time PCR

George M. Blackstone[a,*], Jessica L. Nordstrom[a], Michael C.L. Vickery[a], Michael D. Bowen[b], Richard F. Meyer[b], Angelo DePaola[a]

[a] *Gulf Coast Seafood Laboratory, U.S. Food and Drug Administration, Post Office Box 158, Dauphin Island, AL 36528-0158, USA*
[b] *Centers for Disease Control and Prevention, Atlanta, GA 30333, USA*

Abstract

A real time polymerase chain reaction (PCR) assay was developed and evaluated to detect the presence of the thermostable direct hemolysin gene (*tdh*), a current marker of pathogenicity in *Vibrio parahaemolyticus*. The real time PCR fluorogenic probe and primer set was tested against a panel of numerous strains from 13 different bacterial species. Only *V. parahaemolyticus* strains possessing the *tdh* gene generated a fluorescent signal, and no cross-reaction was observed with *tdh* negative *Vibrio* or non-*Vibrio* spp. The assay detected a single colony forming unit (CFU) per reaction of a pure culture template. This sensitivity was achieved when the same template amount per reaction was tested in the presence of 2.5 μl of a *tdh* negative oyster:APW enrichment (oyster homogenate enriched in alkaline peptone water overnight at 35 °C). This real time technique was used to test 131 oyster:APW enrichments from an environmental survey of Alabama oysters collected between March 1999 and September 2000. The results were compared to those previously obtained using a streak plate procedure for culture isolation from the oyster:APW enrichment combined with use of a non-radioactive DNA probe for detection of the *tdh* gene. Real time PCR detected *tdh* in 61 samples, whereas the streak plate/probe method detected *tdh* in 15 samples. Only 24 h was required for detection of pathogenic *V. parahaemolyticus* in oyster:APW enrichments by real time PCR, whereas the streak plate/probe method required 3 days and was more resource intensive. This study demonstrated that real time PCR is a rapid and reliable technique for detecting *V. parahaemolyticus* possessing the *tdh* gene in pure cultures and in oyster enrichments.
Published by Elsevier Science B.V.

Keywords: *Vibrio parahaemolyticus*; Oyster; Real time PCR

1. Introduction

A current pathogenicity marker of *Vibrio parahaemolyticus*, a leading cause of gastroenteritis from seafood (Mead et al., 1999), is the thermostable direct hemolysin gene (*tdh*) (Thompson and Vanderzant, 1976; DePaola et al., 1990; Kaysner et al., 1990; Nishibuchi et al., 1992; DePaola et al., 2000). Previous methods for the detection of *V. parahaemolyticus* and *tdh* have been restricted to labor and time intensive testing of individual isolates by phenotypic assays, (Thompson and Vanderzant, 1976; Kaysner et al., 1996), which restricts the number of isolates that can

* Corresponding author. Tel.: +1-251-694-4480; fax: +1-251-694-4477.
E-mail address: gblackstone@cfsan.fda.gov (G.M. Blackstone).

0167-7012/03/$ - see front matter. Published by Elsevier Science B.V.
doi:10.1016/S0167-7012(03)00020-4

be obtained and tested from a given sample. This may result in an underestimation of the prevalence of pathogenic (*tdh* positive) *V. parahaemolyticus* in seafoods. With the development of molecular techniques, DNA probes can now be used to detect the *tdh* gene in colonies on a spread plate (McCarthy et al., 2000). This results in a sharp increase in detection of pathogenic *V. parahaemolyticus* in oysters as more colonies that are suspect are tested (Jones and DePaola, 2001 ASM abstract).

An alternative approach for the detection of pathogenic *Vibrio* spp. has been to use polymerase chain reaction (PCR)-based methods to test food and environmental enrichment samples for specific virulence genes, including the cholera toxin gene (*ctx*) of *Vibrio cholerae* (Koch et al., 1993; DePaola and Huang, 1995), and the *tdh* and thermostable related hemolysin genes (*trh*) of *V. parahaemolyticus* (Tada et al., 1992; Bej et al., 1999; McCarthy and Blackstone, 2000). One challenge presented by the use of PCR-based methods is the presence of PCR inhibitors that are prevalent in oysters (Lees et al., 1994). Therefore, these methods often necessitate labor intensive DNA extraction and purification procedures (Bej et al., 1999; McCarthy and Blackstone, 2000) or extensive sample dilution during the enrichment process (DePaola and Huang, 1995).

While conventional PCR-based methods are highly sensitive and specific, they require post-PCR detection procedures, such as gel electrophoresis. Real time PCR eliminates the need for post-PCR processing by measuring the accumulation of PCR amplicons during each cycle of PCR in real time, thus decreasing analytical time and labor. In addition, because fluorescence increases in direct proportion to the amount of specific amplicons, real time PCR can be used for quantitation (Fortin et al., 2001; Kimura et al., 2000; Lyon, 2000; Nogva et al., 2000). Since fluorogenic probes target gene-specific sequences internal to the primer sites, real time PCR imparts an added degree of specificity compared to conventional PCR-based methods.

This study examined the sensitivity and specificity of a real time PCR assay developed on the Smart Cycler® system by Cepheid (Sunnyvale, CA) for the detection of the *V. parahaemolyticus tdh* gene, and evaluated its performance in the detection of *tdh* in environmental oysters compared to a modification of the BAM streak plating method (utilizing a DNA probe to detect the *tdh* gene) (Elliot et al., 1995).

2. Materials and methods

2.1. Preparation of pure culture DNA templates for analysis by real time PCR

The bacterial strains listed in Table 1 were grown overnight at 35 °C in alkaline peptone water (APW) (1% peptone, 1% NaCl) with shaking. A 1-ml aliquot of each overnight bacterial culture was boiled in a snap cap tube for 15 m. Cellular debris was pelleted by centrifugation at 16,000 × *g* for 30 s and the resulting APW supernatant was used as a crude DNA template for PCR (APW was not found to inhibit the PCR). Equal volumes of crude DNA template from each of the individual strains (other than the *tdh* positive strains) were combined to form the DNA pools for the respective species, while the 42 *tdh* positive strains, were assayed individually. A 2.5-μl aliquot from each DNA pool or a 1.0-μl aliquot from the *tdh* positive cultures was used as template in the real time PCR assay described below.

Table 1
Specificity of real time PCR *tdh* assay tested against pooled bacterial DNA

Pooled species	Number tested	*tdh* genotype[a]	Number testing positive by real time PCR
Aeromonas hydrophila	2	–	0
Bacillus subtilis	2	–	0
Escherichia coli	2	–	0
Listeria monocytogenes	5	–	0
Salmonella spp.	2	–	0
Vibrio alginolyticus	2	–	0
Vibrio cholerae	20	–	0
Vibrio fluvialis	16	–	0
Vibrio hollisae	7	+[b]	0
Vibrio metschnikovii	15	–	0
Vibrio mimicus	10	–	0
Vibrio vulnificus	20	–	0
Vibrio parahaemolyticus[c]	12	–	0
Vibrio parahaemolyticus[d]	42	+	42

[a] Determined by alkaline phosphatase DNA probe.
[b] Five strains *tdh* positive; DNA probe and real time PCR conditions optimized to avoid detection.
[c] *tdh* negative strains.
[d] *tdh* positive strains.

2.2. Primers and fluorogenic probe for the detection of tdh positive V. parahaemolyticus by real time PCR

Primer Express Software from Applied Biosystems (Foster City, CA) was used to design oligonucleotide primers and a fluorogenic probe targeting a specific conserved 75 base pair region of the *V. parahaemolyticus tdh*S gene (*V. parahaemolyticus tdh*S, GenBank Accession Number D90101). The forward primer: 5′-AAA CAT CTG CTT TTG AGC TTC CA-3′; and the reverse primer: 5′-CTC GAA CAA CAA ACA ATA TCT CAT CAG-3′, were synthesized by Invitrogen (Baltimore, MD). The fluorogenic probe: 5′-FAM-TGT CCC TTT (T)CC TGC CCC CGG-3′ was provided by the Centers for Disease Control Core Synthesis Facility (Atlanta, GA). FAM represents the fluoroscein reporter and (T) is the nucleotide attachment site of the TAMRA quencher molecule to the oligonucleotide.

2.3. Real time PCR assay for the detection of tdh positive V. parahaemolyticus

Real time PCR amplification was run in a 25-μl volume using the following reaction components (final concentrations shown): 1× PCR Amplification Buffer [10× buffer consisted 200 mM Tris–HCl (pH 8.4) and 500 mM KCl] (Invitrogen), 9 mM $MgCl_2$ (additional optimization showed that the $MgCl_2$ concentration could be reduced to 6 mM with equivalent results), 200 nM of each of the dNTPs (Roche, Indianapolis, IN), 300 nM of each of the primers described above, 50 nM fluorogenic probe described above, 1.25 U Platinum™ *Taq* polymerase (Invitrogen), and either 1.0 or 2.5 μl of template (see Sections 2.1, 2.4 and 2.6)). Real time PCR thermal cycling was run using the Smart Cycler® system from Cepheid utilizing the following parameters: 94 °C initial hold for 2 m to denature the DNA and activate the hot start *Taq* polymerase, followed by 50 cycles of amplification, with each amplification cycle consisting of denaturation at 94 °C for 10 s followed by a combined primer annealing/extension step at 60 °C for 12 s. The accumulated fluorescence in each Smart Cycler® reaction tube was measured at the end of each amplification cycle by the instrument, with positive samples generating a signal of at least 30 fluorescent units above baseline within 50 cycles. A positive control of TX2103 (*tdh* positive clinical strain isolated during a 1998 outbreak in Texas) and a negative control (dH_2O as template in the reaction) were prepared for each PCR master mix.

2.4. Standard curve for real time PCR assay

A 6-h mid-log phase culture in APW of the *V. parahaemolyticus* TX 2103 strain was diluted serially tenfold (with the highest dilution at 10^{-8}) in sterile phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}7H_2O$, 1.4 mM KH_2PO_4, pH 7.3 ± 0.2). Duplicate spread plates of each dilution were spread plated onto T_1N_3 media (1% tryptone, 3% NaCl, 2% agar), and a 1-ml aliquot of the 6 h culture and each serial dilution were used to prepare DNA template by boiling (Section 2.1) as described above. Viable plate counts were obtained after an overnight incubation at 35 °C. Aliquots (1.0 μl) of each boiled cell dilution were used as template for real time PCR, in order to produce a standard curve for examination of the sensitivity and linearity of the assay over the template dilution range. To produce the standard curve, the log value of the calculated number of cells added to each reaction tube (derived from plate counts) was plotted vs. the cycle threshold (Ct) value. The Ct value may be defined as "the first cycle at which there is a significant increase in fluorescent signal over the background signal or a specified threshold" (Smart Cycler® Operator Manual, 2001, Cepheid). Inhibition of the PCR reaction by oyster homogenate was tested using a standard curve with a pure culture template (as described above), except an additional 2.5 μl from a *tdh* negative oyster enrichment was also added to each reaction tube. This oyster enrichment was previously tested by DNA probe and real time PCR and was found to be negative for *tdh* (Sections 2.6 and 2.7). The difference in Ct values between the *tdh* template alone added to the reaction, and the *tdh* template added along with oyster enrichment showed the inhibitory effect of oyster homogenate (Fig. 2).

2.5. Preparation of oyster samples

Sample collection, preparation, and enrichment conditions were as previously described (Nordstrom and DePaola, 2002). Briefly, a sample comprised of

10–12 oysters (200–250 g) was diluted 1:1 in APW and blended for 90 s. A 50-ml aliquot of blended oyster homogenate was added to 200 ml of APW (resulting in a final 1:10 dilution of oyster homogenate) and enriched overnight at 35 °C. For each sample, a 10-ml aliquot of oyster enrichment (1:10 oyster in APW incubated overnight at 35 °C) was frozen at −20 °C until utilized for analysis by real time PCR. Thiosulfate–citrate–bile salts–sucrose (TCBS) plates, a selective and differential agar were then streaked from the 1:10 oyster enrichment and incubated overnight at 35 °C. The next day up to 48 suspect green (sucrose negative) colonies were picked and each was inoculated into 100 μl of APW in a 96-well plate. The 96-well plate was then incubated 4–6 h and replicated to a VVA plate [a differential agar (Kaysner and DePaola, 2001)], using a 48 prong replicator (Boekel Scientific, Feasterville, PA).

2.6. Analysis of oyster samples for the presence of tdh positive V. parahaemolyticus by real time PCR

After first thawing, the oyster:APW enrichment at room temperature, template for the real time PCR assay was prepared by boiling 1 ml of the enrichment for 15 m and then briefly centrifuging in a tabletop centrifuge to pellet the cellular debris; 2.5 μl of the supernatant was used as template for real time PCR.

2.7. Analysis of oyster samples for the presence of tdh positive V. parahaemolyticus by the streak plate/probe method

The colonies on the VVA plate were lifted using Whatman 541 filters. This allowed testing of 48 suspect colonies for the presence of the *tdh* gene using the colony lift and DNA hybridization procedure previously described by McCarthy et al., 2000 (Fig. 1). Briefly, the colonies on the filters were lysed using an alkaline lysis solution, and then fixed to the filter by drying in a microwave. The filters were then treated with Protinase K, washed in a salt solution and then hybridized with an alkaline phosphatase labeled DNA probe specific for the *tdh* gene. The hybridized filter was then washed to remove any unbound probe, and then placed in a NBT/BCIP color development solution. Isolates were recovered from colonies which gave a positive signal with the *tdh* DNA probe. These isolates were then tested with API® (bioMérieux, Durham, NC) to ensure they were *V. parahaemolyticus*.

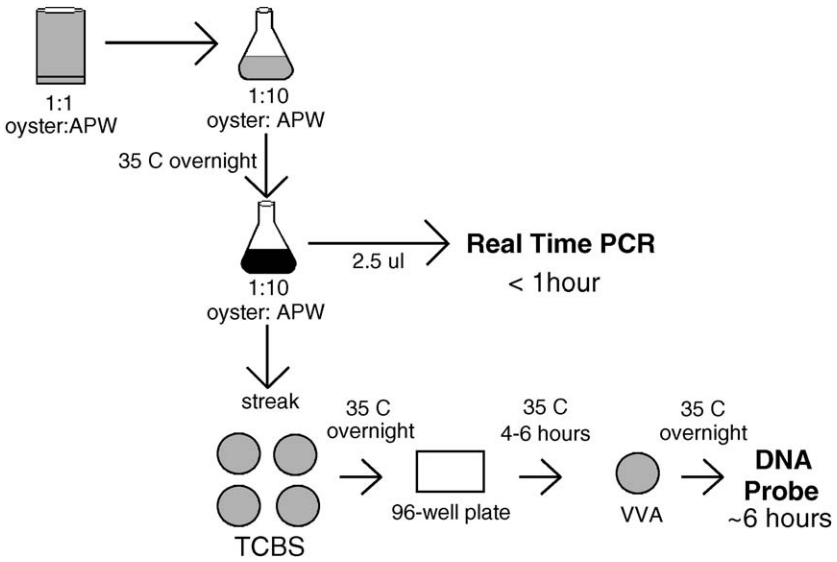

Fig. 1. Schematic of the microbiological sample preparation for analysis by real time PCR and the streak plate/probe method. The total time required for real time PCR is <24 h and the streak plate/probe method requires about 72 h.

3. Results

3.1. Real time PCR assay specificity, sensitivity, and standard curve

Pooled DNA from bacterial strains, shown by DNA probe to lack the *tdh* gene, was tested by real time PCR. None of the pools produced a signal from the *tdh* real time assay. Only bacterial strains possessing the *tdh* gene produced a fluorescent signal (Table 1). Using boiled tenfold serial dilutions of DNA in PBS as template, detection of a single colony forming unit (CFU) was obtained within 38 cycles (Fig. 2). The standard curve showed a linear relationship between the log cell numbers and Ct values across at least six orders of magnitude (10^5 to 10^0 cells per reaction) with a strong correlation coefficient ($r^2 = 0.99$) (Fig. 2). The addition of 2.5 μl *tdh* negative oyster enrichment supernatant inhibited the assay by an average of 4.6 Ct values at each dilution; however, detection of a single *tdh* positive CFU still occurred within 45 cycles (Fig. 2).

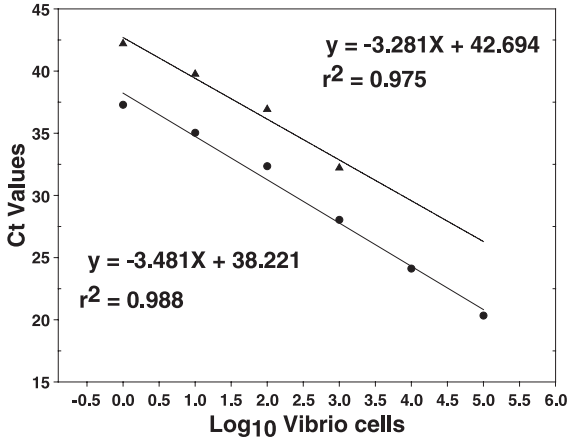

Fig. 2. Standard curves showing the log cell number plotted vs. the real time PCR cycle threshold for 10 fold dilutions of boiled *V. parahaemolyticus* cells diluted to 1 CFU per reaction. The assay showed a linear relationship between the log cell number and Ct value across at least six orders of magnitude (10^5 to 10^0 cells per reaction) with a strong correlation coefficient ($r^2 = 0.988$). The addition of 2.5 μl *tdh* negative oyster enrichment supernatant inhibited the assay by an average of 4.6 Ct values at each dilution; however, detection of a single *tdh* positive CFU was still achieved. (●) Cells in PBS only; (▲) cells in PBS plus oyster enrichment.

Table 2
Detection of *tdh* gene in 131 oyster enrichments by real time PCR and by streak plate/probe method

	Enrichments positive by the streak plate/probe method	Enrichments negative by the streak plate/probe method	Totals
Enrichments positive by real time PCR	12	49	61
Enrichments negative by real time PCR	3	67	70
Totals	15	116	131

3.2. Analysis of oyster samples for the presence of tdh positive V. parahaemolyticus by real time PCR and by streak plating/DNA probe

A total of 131 oyster enrichment samples was tested for the presence of *tdh* positive strains of *V. parahaemolyticus* by both real time PCR and the streak plate/probe method. Twelve samples tested positive by both methods, while 67 samples tested negative by both methods. Forty-nine samples tested positive by PCR that were negative by DNA probe, while three samples tested negative by PCR that were positive by DNA probe (Table 2). A significantly higher detection rate ($p < 0.05$, McNemar's analysis; Fleiss, 1981) was obtained with real time PCR.

4. Discussion

Recently, real time PCR has been shown to be useful in the detection of pathogenic bacteria in food products (Kimura et al., 2000; Lyon, 2000; Nogva et al., 2000; Fortin et al., 2001). This is the first report demonstrating the efficacy of real time PCR for the detection of pathogenic (*tdh* positive) *V. parahaemolyticus* in oyster enrichments. The primer set and probe sequences used were selected from regions of the *V. parahaemolyticus tdh* gene sequence that are highly specific to this gene, and were located downstream from the region targeted by the DNA probe used in the streak plate/probe method. Use of the fluorescent probe in real time PCR provided an additional level of assay specificity that was demonstrated using a variety of non-vibrio species as well as numerous

other vibrio species including *V. hollisae*, *V. mimicus*, and *V. cholerae* non-O1, of which certain strains can contain hemolysin genes with close homology to *tdh* (Nishibuchi and Kaper, 1995). McCarthy et al. reported that hybridization modifications were required in order to eliminate detection of *tdh* positive *V. hollisae* with the DNA probe, and that the two species were indistinguishable when using conventional PCR. None of the strains from these related vibrio species or any other non-vibrio bacterial species tested generated a real time amplification signal. A single CFU in the real time PCR reaction provided adequate target for detection of the *tdh* gene within 1 h. The dynamic range of detection (10^5 to 10^0) demonstrates that the assay retains its sensitivity over a broad range of template DNA concentrations. While the PCR reaction was delayed four to five cycles when running the oyster enrichment matrix (compared to PBS), no extraction or purification steps were required in order to obtain a single CFU limit of detection. In addition, since no post-PCR detection procedures were required, the real time PCR procedure was much more rapid (1 vs. 4 h) than conventional methods.

The strong correlation ($r^2 = 0.99$) between the number of initial *tdh* targets (CFU) in the PCR reaction and the associated Ct values illustrates the quantitative potential for real time PCR. While this is not relevant to the screening of enrichments, real time PCR may be useful in the quantitation of high *V. parahaemolyticus* levels initially present in environmental or seafood samples. In addition, sample concentration of unenriched samples prior to the assay may aid in utilizing real time PCR for the rapid detection and possible quantitation of pathogenic *V. parahaemolyticus* and other organisms from seafood samples, an area we are currently exploring.

The detection rate of *tdh* in the oyster enrichments was significantly higher ($p < 0.05$) with real time PCR (47%) than with the streak plate/probe method (11%), and was considerably higher than was observed in previous oyster surveys (DePaola et al., 1990, 2000; Kaysner et al., 1990; Cook et al., 2001). The higher detection rate might be attributed to the testing of a greater number of potential *tdh* positive cells by real time PCR than by the testing of colonies (each representing only a single cell from the enrichment) with the DNA probe. In an oyster:APW enrichment, *V. parahaemolyticus* levels typically reach 10^8 cells per ml (unpublished results). Thus, a 2.5-μl portion of the enrichment used as PCR template, would test greater than 10^5 *V. parahaemolyticus* cells in a single reaction. The ability to replicate only six columns of a 96-well plate (resulting in a maximum of 48 colonies onto a standard petri dish) limits the practical testing of cells by the streak plate/probe method. In addition, since the oyster enrichment used for the streak plate/probe method is plated onto TCBS, a selective and differential medium, the growth of some strains of *V. parahaemolyticus* (possibly possessing the *tdh* gene) may have been inhibited as compared to the non-selective APW enrichment which was tested by real time PCR. Recently, a modification of the streak plate/probe method allowed the testing of approximately 1000 colonies per sample, and improved the rate of detection to near 50% (Nordstrom and DePaola, 2002). However, spread plating with colony lift and filter hybridization is still much more resource intensive than real time PCR.

The storage of oyster:APW enrichments at −20 °C before boiling and analysis by PCR may have adversely affected the real time PCR reaction. For some samples, an increase in Ct values was observed after prolonged storage at −20 °C (>3 months) (data not shown); this was possibly the result of degradation of the target DNA during freezing/thawing, perhaps by DNase activity. An underestimation of the prevalence of *tdh* in these samples may have resulted from this target DNA degradation. This might offer an explanation as to why the real time assay did not detect *tdh* in three of the samples that tested positive by the streak plate/probe method. Boiling of the enrichments before freezing appears to increase the sensitivity of the real time assay, probably by denaturation/damage of any DNA-degrading enzymes. A variation in levels of PCR inhibitors from sample to sample may be another possible explanation for why real time PCR did not detect *tdh* in these three samples. These hypotheses will require further experimentation for validation. It is worth noting that the species identification of all isolates recovered was determined by API® to be *V. parahaemolyticus*; however, for the three samples which were positive by probe and negative by PCR, a culture was recovered from only one sample. This isolate was confirmed as *V. parahaemolyticus*.

This study demonstrated that the real time PCR assay is a highly specific (detected only *tdh* positive *V.*

parahaemolyticus strains), sensitive (1 CFU per reaction limit of detection), rapid (time taken was <24 h for total assay and <1 h for detection), and efficient method for the detection of pathogenic *V. parahaemolyticus* in oyster enrichments.

Acknowledgements

The authors would like to acknowledge the technical assistance of Greer Kaufman, as well as the assistance of K.R. Calci in the preparation of this manuscript.

References

Bej, A.K., Patterson, D.P., Brasher, C.W., Vickery, M.C.L., Jones, D.D., Kaysner, C.A., 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tlh, tdh* and *trh*. J. Microbiol. Methods 36, 215–225.

Cook, D.W., O'Leary, P., Hunsucker, J.C., Sloan, E.M., Bowers, J.C., Blodgett, R.J., DePaola, A., 2001. *Vibrio vulnificus* and *Vibrio parahaemolyticus* in retail shell oysters: a United States survey June 1998–July 1999. J. Food Prot. 65, 79–87.

DePaola, A., Huang, G., 1995. Effect of dilution, incubation time, and temperature of enrichment on cultural and PCR detection of *Vibrio cholerae*. Mol. Cell. Probes 9, 75–81.

DePaola, A., Hopkins, L.H., Peeler, J.T., Wentz, B., McPhearson, R.M., 1990. Incidence of *Vibrio parahaemolyticus* in U.S. coastal waters and oysters. Appl. Environ. Microbiol. 56, 2299–2302.

DePaola, A., Kaysner, C.A., Bowers, J.C., Cook, D.W., 2000. Environmental investigations of *Vibrio parahaemolyticus* in oysters following outbreaks in Washington, Texas, and New York (1997, 1998). Appl. Environ. Microbiol. 66, 4649–4654.

Elliot, E.K., Kaysner, C.A., Jackson, L., Tamplin, M.L., 1995. *Vibrio cholerae, V. parahaemolyticus, V. vulnificus,* and other *Vibrio* spp. U.S. Food and Drug Administration Bacteriological Analytical Manual. A.O.A.C. International, Gaithersburg, MD, pp. 9.01–9.27.

Fleiss, J.L., 1981. Statistical Methods for Rates and Proportions, 2nd ed. Wiley, New York.

Fortin, N.Y., Mulchandani, A., Chen, W., 2001. Use of real-time polymerase chain reaction and molecular beacons for the detection of *Escherichia coli* O157: H7. Anal. Biochem. 289, 281–288.

Jones, J.L., DePaola, A., 2001. Improved recovery of *Vibrio parahaemolyticus* from oysters using colony hybridization following enrichment. Poster P-97. ASM 101st General Meeting.

Kaysner, C.A., DePaola Jr., A., 2001. *Vibrio*. In: Downes, F.P., Ito, K. (Eds.), Compendium of Methods for the Microbiological Examination of Foods. American Public Health Association, Washington, DC, pp. 405–420.

Kaysner, C.A., Abeyta, C.J., Stott, R.F., Lilja, J.L., Wekell, M.M., 1990. Incidence of urea-hydrolyzing *Vibrio parahaemolyticus* in Willapa Bay, Washington. Appl. Environ. Microbiol. 56, 904–907.

Kaysner, C.A., Abeyta, C.J., Scott, R.F., Krane, M.H., Wekell, M.M., 1996. Enumeration of *Vibrio* species, including *V. cholerae* from samples of an oyster growing area, Grays Harbor, Washington. J. Food Prot. 53, 300–302.

Kimura, B., Kawasaki, S., Nakano, H., Fujii, T., 2000. Rapid, quantitative PCR monitoring of growth of *Clostridium botulinum* type E in modified-atmosphere-packaged fish. Appl. Environ. Microbiol. 67, 206–216.

Koch, W.H., Payne, W.L., Wentz, B.A., Cebula, T.A., 1993. Rapid polymerase chain reaction method for detection of *Vibrio cholerae* in foods. Appl. Environ. Microbiol. 59, 556–560.

Lees, D.N., Henshilwood, K., Dore, W.J., 1994. Development of a method for detection of enteroviruses in shellfish by PCR with poliovirus as a model. Appl. Environ. Microbiol. 60, 2999–3005.

Lyon, W.J., 2000. TaqMan PCR for detection of *Vibrio cholerae* O1, O139, non-O1, and non-O139 in pure cultures, raw oysters, and synthetic seawater. Appl. Environ. Microbiol. 67, 4685–4693.

McCarthy, S.A., Blackstone, G.M., 2000. DNA isolation methods for detection of pathogenic *Vibrio parahaemolyticus* by PCR. Lab. Inform. Bull., vol. 4222(16). Department of Health and Human Services, Rockville, MD, pp. 1–13.

McCarthy, S.A., DePaola, A., Kaysner, C.A., Hill, W.E., Cook, D.W., 2000. Evaluation of non-isotopic DNA hybridization methods for detection of the *tdh* gene of *V. parahaemolyticus*. J. Food Prot. 63, 1660–1664.

Mead, P.S., Slutsker, L., Dietz, V., McGaig, L.F., Bresee, J.S., Sharpiro, C., Griffin, P.M., Tauxe, R.V., 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5, 607–625.

Nishibuchi, M., Kaper, J., 1995. Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. Infect. Immun. 63, 2093–2099.

Nishibuchi, M., Fasano, A., Russell, R.G., Kaper, J.B., 1992. Enterotoxigenicity of *Vibrio parahaemolyticus* with and without genes encoding thermostable direct hemolysin. Infect. Immun. 60, 3539–3545.

Nogva, H.K., Rudi, K., Naterstad, K., Holck, A., Lillehaug, D., 2000. Application of 5′-nuclease PCR for quantitative detection of Listeria monocytogenes in pure cultures, water, skim milk, and unpasteurized whole milk. Appl. Environ. Microbiol. 66, 4266–4271.

Nordstrom, J.L., DePaola, A., 2002. Improved recovery of pathogenic *Vibrio parahaemolyticus* from oysters using colony hybridization following enrichment. J. Microbiol. Methods, 52, 273–277.

Smart Cycler® Operator Manual, 2001. p. 21. Chap. 3.1.

Tada, J., Ohashi, T., Nishimura, N., Shirasaki, Y., Ozaki, H., Fukushima, S., Takano, J., Nishibuchi, M., Takeda, Y., 1992. Detection of the thermostable direct hemolysin gene (*tdh*) and the thermostable direct hemolysin-related hemolysin gene (*trh*) of Vibrio parahaemolyticus by polymerase chain reaction. Mol. Cell. Probes 6, 477–487.

Thompson, C.A., Vanderzant, C., 1976. Serological and hemolytic characteristics of *Vibrio parahaemolyticus* from marine sources. J. Food Sci. 41, 204–205.