Proposal Subject:	Alternative Analytical Method for *Vibrio vulnificus*
Specific NSSP Guide Reference:	Section IV. Guidance Documents, Chapter II. Growing Areas .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods (5) Interim Approval by ISSC Executive Board August 2007
Text of Proposal/ Requested Action	Accept the adoption of Quantitative Real-Time PCR as an alternative analytical protocol to determine the levels of *Vibrio vulnificus*. Rename "Sec IV Chapter II.10 (5) Interim Approval by ISSC Executive Board August 2007" to reflect the methods committee's action on these methods and to include QPCR as an alternative method for analysis of Vv in PHP products
Public Health Significance:	Improve the speed of analysis to help the industry to increase the amount of PHP products in the market.
Cost Information (if available):	See attached application report
Action by 2009 Laboratory Methods Review Committee	Recommended acceptance of Proposal 09-103 SYBR Green 1 QPCR-MPN in conjunction with the PHP of oysters as a Type IV method provided the information on the step-by-step procedures and the manufacturer's ruggedness data are submitted within 30 days.
Action by 2009 Task Force I	Recommended adoption of Proposal 09-103 as submitted.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force I on Proposal 09-103.
Action by USFDA 02/16/2010	Concurred with Conference action on Proposal 09-103.

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	Sybr Green I QPCR-MPN for Rapid Detection of *Vibrio vulnificus*
Name of the Method Developer	Anita Wright et. Al.
Developer Contact Information	Anita Wright 461 AFPL bldg. Newell Dr. Gainesville, FL 32611 352-392-1991 ext. 311

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	Give the shellfish industry, regulatory and analytical labs an alternative method to confirm upern bacteria in shellfish
2. What is the intended purpose of the method?	Y	Replace confirmation step in MPN determination of Vibrios in shellfish
3. Is there an acknowledged need for this method in the NSSP?	Y	End users are requiring faster more economical alternatives to the current approved method
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Quantitative PCR
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	
Method Scope	Y	
References	Y	
Principle	Y	
Any Proprietary Aspects	Y	
Equipment Required	Y	
Reagents Required	Y	
Sample Collection, Preservation and Storage Requirements	Y	
Safety Requirements	Y	
Clear and Easy to Follow Step-by-Step Procedure	Y	
Quality Control Steps Specific for this Method	Y	
C. Validation Criteria		
1. Accuracy / Trueness	Y	
2. Measurement Uncertainty	Y	
3. Precision Characteristics (repeatability and reproducibility)	Y	
4. Recovery	n/a	
5. Specificity	Y	
6. Working and Linear Ranges	Y	
7. Limit of Detection	Y	
8. Limit of Quantitation / Sensitivity	Y	
9. Ruggedness	Y	
10. Matrix Effects	Y	

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	
D. Other Information		
1. Cost of the Method	Y	
2. Special Technical Skills Required to Perform the Method	Y	
3. Special Equipment Required and Associated Cost	Y	
4. Abbreviations and Acronyms Defined	N/A	
5. Details of Turn Around Times (time involved to complete the method)	Y	
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	
Submitters Signature	Date:	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments: See attached application document.		

DEFINITIONS

1. **Accuracy/Trueness** - Closeness of agreement between a test result and the accepted reference value.
2. **Analyte/measurand** - The specific organism or chemical substance sought or determined in a sample.
3. **Blank** – Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. **Comparability** – The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. **Fit for purpose** – The analytical method is appropriate to the purpose for which the results are likely to be used.
6. **HORRAT value** – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. **Limit of Detection** – the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. **Limit of Quantitation/Sensitivity** – the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. **Linear Range** – the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. **Measurement Uncertainty** – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. **Matrix** – The component or substrate of a test sample.
12. **Method Validation** – The process of verifying that a method is fit for purpose.[1]
13. **Precision** – the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. **Repeatability** – the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. **Reproducibility** – the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. **Quality System –** The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision–making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. **Recovery –** The fraction or percentage of an analyte or measure and recovered following sample analysis.
16. **Ruggedness** – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** – the ability of a method to measure only what it is intended to measure.[1]
18. **Working Range –** the range of analyte or measure and concentration over which the method is applied.

REFERENCES:

7. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
8. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
9. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.

10. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
11. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
12. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

QPCR-MPN Assay for Rapid Detection of *Vibrio vulnificus* in Oysters

Justification for New Method

This proposal was prepared to support the use of a new molecular detection method: Sybr Green I QPCR-MPN for Rapid Detection of *Vibrio vulnificus* to be substituted for the use of DNA probe colony hybridization for confirmation of the presence of *V. vulnificus* growth in the MPN enrichment protocol described in the FDA Bacteriological Analytical Mannual (8). This protocol is submitted for approval to the Laboratory Methods Review Committee. Method was developed by collaborative efforts of Dr. Anita Wright, Dr. Steve Otwell, Victor Garrido, Charlene Burke, and Melissa Evans, University of Florida, Gainesville, Florida.

Developer Contact Information:
Anita Wright, Ph.D. (Method Developer)
461 Aquatic Food Products Building
Newell Drive
Gainesville, Florida
352-392-1991 x 311

Date of Submission
Proposal submission date is June 30, 2009.

Purpose and Intended Use of the Method
V. vulnificus the leading cause of death in the US related to seafood consumption and is predominantly associated with consumption uncooked Gulf Coast oysters (7). The proposed method will benefit the seafood industry and the consumer by providing improved, faster, and more accurate deteiction of this pathogen in oysters. This method is being proposed for use in validation of Post Harvest Processing protocols, as well as for future applications to assure the public of a safer product.

Need for the New Method in the NSSP
V. vulnificus QPCR-MPN is proposed as an alternative to the standard MPN assay using most probable number (MPN) end-point titration of replicate samples in enrichment broth cultures (4). In the current protocol, species-specific growth in enrichment is determined by isolating typical *V. vulnificus* colonies on selective medium with subsequent confirmation by DNA probe (15). This method is laborious cost prohibitive, labor intensive, and time consuming (6, 8). Furthermore, users of this protocol have expressed difficulty with DNA probe product reliability and plating problems related to "spreading" colonies that interfer with the assay. Total amount of time to perform the traditional MPN method with DNA colony blot hybridization as a confirmatory method is at least 4 days, with numerous steps; additionally, technician requires a great deal of experience in performing this assay for successful quantification to be possible. QPCR-MPN method reduces working time half and offers greater sensitivity for detection of *V. vulnificus*; with detection of 1 bacterium per gram post enrichment in alkaline peptone water (APW) overnight (1, 4, 9, 10, 11, 16).

Although these post harvest processing methods are currently employed on < 10% of all domestic raw oyster sales in the United States, the industry continues to examine and employ new technologies and take initiative on expanding acceptance and knowledge regarding these treated oyster products (5). The industry is investing money and resources to ensure a market acceptance by educated oyster public, in addition to mitigating risk potential for the at risk consumers of fresh oysters. ISSC mandated that 25% of oysters harvested from the Gulf of Mexico receive some type of validated post harvest processing. Thus, there is a continued need for improve validation methods.

The University of Florida has partnered with several dealers who are using ISSC recommended for validation of post-harvest processing methods. Work was performed in 2004, working with Leavin's seafood (nitrogen

freezing) and Tommy Ward Seafood (blast freezing). Throughout the validation, samples were randomly selected for side-by-side comparisons of standard MPN (FDA BAM) to QPCR-MPN. Test results support the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster PHP, which was described in a publication by Wright et al., 2007 (Appendix 3).

QPCR-MPN method increased assay sensitivity and reduced both time and labor costs. Detection of *V. vulnificus* was achieved at levels < 30 CFU/g as required for validation protocols (2, 10, 15). For these reasons we propose acceptance of the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster post harvest processing. The oyster industry's livelihood will be determined by their ability to adapt to FDA demands, and evolving technological breakthroughs. The scientific community works to discover the most expedient, accurate detection methods and the most effective treatments for the eradication of naturally occurring Vibrio as the public continues to demand a fresh, uncooked product. Until this demand has abated, the industry and the scientific community will continue to work in conjunction to learn more and thus protect the public from Vibrio disease.

Method Limitations and Potential Indications of Cases Where the Method May Not Be Applicable to Specific Matrix Types

This method is specific to applications testing growth of *V. vulnificus* in MPN enrichment of oyster homogenates at concentrations of 1.0 g or less. This QPCR method does not claim to differentiate between pathogenic and nonpathogenic *V. vulnificus*. QPCR-MPN provides more sensitive detection than standard MPN, as enriched samples that were PCR positive but negative on selective media were falsely negative on mCPC, as indicated by agreement of positive mCPC and QPCR results in more diluted inocula of the same sample (15). The result is an increase in sensitivity and a reduction in time and labor costs while still permitting detection of *V. vulnificus* at levels < 30 CFU/g as required for validation protocols (2, 10, 15). For these reasons we propose acceptance of the application of QPCR-MPN for improved assessment of validation and verification protocols related to oyster post harvest processing.

Method Documentation

Method Title

Sybr Green I QPCR-MPN for Rapid Detection of *Vibrio vulnificus*

Method Scope

This method is designed for MPN analysis of validation trials for oyster PHP.

Principle

QPCR-MPN will be substituted as an alternative to the officially recognized NSSP method for MPN analysis of validation trials for oyster PHP (3). Specifically QPCR will be substituted for microbiological/DNA probe confirmation of *V. vulnificus* growth in MPN enrichment. Since the FDA and the ISSC have mandated postharvest processing (PHP) of oysters harvested from Gulf Coast states in order to reduce *V. vulnificus* infections validation and verification are necessary in order to ensure that the process will substantially reduce numbers of *V. vulnificus* bacteria to levels below the predicted threshold for disease. QPCR-MPN is a rapid and reliable method to accomplish agency mandates and industry goals.

Proprietary Aspects

Ingredients in Smartmix beads (Cepheid©) containing PCR reagents for use with Cepheid© Smartcycler are proprietary information.

Equipment

Cepheid© Smartcycler

Reagents

- SmartMix beads (Cepheid)
- SYBR green I (Invitrogen)
- VvhA forward and reverse primers (Geno-mechanix, Gainesville, FL)
- Autoclaved molecular grade water

Media (Media are specified in FDA BAM, reference 8)

- Modified colistin polymyxin cellobiose (mCPC) agar
- T1N1 agar
- Alkaline peptone water (APW) enrichment broth
- Phosphate buffered saline (PBS)

Matrix or Matrices of Interest

The validation of post harvest processing for raw gulf coast oysters is performed on oyster homogenate. Thus the matrix is dilutions of oyster homogenate, consisting of oyster meats and PBS.

Sample Collection, Preservation, Preparation, Storage, Cleanup, Test Procedures

- A boiling lysis was used for DNA preparation. APW cultures (1 ml) were centrifuged (15,000 x g,10 min), resuspended in 1 ml phosphate-buffered saline (PBS, boiled for 10 min, and subsequently centrifuged to removeparticulates. Supernatants were stored at -20°C.
- VvhA Primers (Geno-mechanix, Gainesville, FL) were stored at -20°C.
- DNA templates (2 µl) and water were added to QPCR reactions for a total volume of 25 µl.

Cost of the Method

The cost of the method varies depending on the Q-PCR platform chosen; however, the Cepheid© smartcycler platform costs approximately $5 per PCR reaction.

Special Technical Skills Required to Perform the Method

Only basic laboratory skills are required.

Special Equipment Required and Associated Cost

Equipment	Approximate Cost
Cepheid© thermocycler	$30,000 + accessories
Incubator	$3,000 - $6,000
Centrifuge	$2,000
Heat block	$500

Abbreviations and Acronyms

- PHP –post harvest processing
- DNA- deoxyribonucleic acid
- QPCR- quantitative polymerase chain reaction
- APW- alkaline peptone water
- PBS- phosphate buffered saline
- MPN- most probable number
- VVAP- Vibrio vulnificus alkaline-phosphatase probe
- mCPC- modified colistin polymyxin cellobiose

Test Procedures and Quality Control

MEDIA:

Dehydrated media is commercially dehydrated. Media must be sterilized according to manufacturer's instructions. Prepared culture media, dehydrated media and media components must be stored in a cool, clean, dry space unless refrigeration is required as per manufacturer instruction. Stored media is labeled with batch number, expiration date and sterilization date. Storage of prepared culture media at room temperature does not exceed 7 days. Refrigerated storage of prepared media with loose fitting closures does not exceed 1 month; screw-cap closures do not exceed 3 months. All prepared media stored under refrigeration are held at room temperature overnight prior to use.

To determine the pH of prepared media, a pH meter with a standard accuracy of 0.1 units is used. The pH meter is calibrated with each use and a minimum of two standard buffer solutions (ph 4, 7 and 10) are used to calibrate the pH meter. Standard buffer solutions are used once and discarded.

COLD STORAGE:

Refrigerator temperature must be monitored daily; temperature is maintained between 0˚C to 4˚C. Freezer temperature must be monitored at least once daily, freezer temperatures is maintained at -20˚C (DNA storage) and –80˚C (strain storage).

INCUBATOR:

Temperature of incubators must be maintained at 30˚C (+/-0.5), 37˚C (+/-0.5), and 40˚C (+/-0.5). Thermometers must be graduated no greater than 0.5˚C increments. Temperatures are taken twice daily.

SUPPLIES:

Utensils and containers made of clean borosilicate glass, stainless steel or other non-corroding material. Culture tubes made of a suitable size to accommodate the volume for broth and samples. Sample containers made of glass or other inert material.

Dilution bottles and tubes are made of plastic and closed with attached snap-lock lids. Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes. Reusable sample containers must be capable of being properly washed and sterilized.

Hardwood applicator transfer sticks, utilized for streaking and picking positive colonies, and Whatman # 3 and #541 filter papers, utilized in colony blot hybridization, are sterilized prior to use and stored in sterile, airtight containers.

Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10ml are not used to deliver 1ml; nor, are pipettes larger than 1ml used to deliver 0.1ml.

MAINTENANCE:

Routine autoclave maintenance must be performed and serviced annually or as needed by a qualified technician and records maintained. Autoclave provides a sterilizing temperature of 121˚C (tolerance 121 +/- 2˚C) as determined daily. Spore suspensions or strips must be used monthly to evaluate the effectiveness of the autoclave sterilization process, with results recorded. Heat sensitive tape must be used with each autoclave batch. Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature must be maintained in an autoclave log.

SHELLSTOCK SAMPLES:
A representative sample of shellstock is collected. Shellstock is collected in clean, waterproof, puncture resistant containers. Shellstock labeled with collector's name, type of shellstock, the source, the harvest area, time, date and place of collection. Shellstock are maintained in dry storage between 0 and 10˚C until examined. Examination of the sample is initiated as soon as possible after collection, and does not exceed 24 hours after collection.

Shucking knives, scrub brushes and blender jars are sterilized for 35 minutes prior to use. Blades of shucking knives free from debris corrosion. Prior to scrubbing and rinsing debris off shellstock, the hands of the technician are thoroughly washed with soap and water. Shellstock are scrubbed with a stiff, sterile brush and rinsed under water of drinking water quality. Shellstock are allowed to drain in a clean container or on clean towels prior to opening. Prior to opening, the technician washes hands and rinses with 70% alcohol. Shellstock are not shucked directly through the hinge.

FDA-MPN PREPARATION AND METHOD:
Contents of shellstock are shucked into a sterile, tared blender jar. At least 12 animals (100 g of meat) are used for analysis. The sample is weighted to the nearest 0.1 gram and an equal amount by weight of sterile PBS diluent is added. Samples are blended at high speed for 90 seconds.

Immediately after blending, the homogenized sample is diluted in a multiple dilution series with 3 replicas and inoculated into tubes of APW presumptive media for MPN analysis. Positive and negative controls cultures accompany samples throughout the procedure. Inoculated media are incubated at 37 +/- 0.5˚C. Presumptive tubes are read at 24+/- 2 hours of incubation and transferred if positive. Transfers are made to mCPC plates by sterile hardwood applicator sticks from presumptive positive APW tubes and confirmed by DNA probe.

QPCR-MPN PREPARATION:
Prior to DNA extraction and preparing Cepheid© unit for QPCR, all micro-centrifuge tubes and pipette tips are sterilized for 35 minutes. The technician's hands are washed with soap and water. Gloves are worn and rinsed with 70% alcohol. All Pipetteman and Eppendorf pipettes are calibrated semi-annually and prior to use are wiped down with 70% alcohol. All working areas, centrifuge racks, and equipment are wiped down with 70% alcohol. Proper sterile technique is observed throughout the procedure to ensure contamination free samples.

1ml of sample from each positive MPN tube is used for the boil extraction procedure (appendix 1) to extract DNA to be used as template for Sybr green 1 QPCR-MPN assay as described in appendix 2. Cepheid© thermocycler cycle threshold is set at 30 and factory default is utilized for melt curve analysis regarding peak height.

Validation Criteria

Ruggedness of Assay

Validity of MPN assay for detection of *V. vulnificus* has been previously established by ISSC and FDA. The ruggedness of reagents used for PCR is determined by manufacturer and meet specifications. Method uses a bead format that incorporates all reagents on bead to eliminate common pipetting and cross-contamination errors.

Data Comparability and Statistical Analysis

During 2004 summer PHP validation trials were conducted by The University of Florida Aquatic Food Products group in a partnership with the oyster industry in Apalachicola FL. Side by side field trials compared the FDA-MPN (8), which consists of selection of typical colonies on mCPC and confirmation by VVAP DNA probe, to the QPCR-MPN assay described herein. Results of a side by side sample comparison support application of QPCR technology for validation oyster processing protocols.

Quantitative PCR was applied to most probable number (QPCR-MPN) for validation of PHP methods for reduction of *V. vulnificus in* oysters for Day 1 and Day 7. Published results by Wright et al., 2007 showed that immediately following inoculation of APW (pre-enrichment with either 0.1 or 0.01 g oyster homogenate detection *V. vulnificus* was 100 to 1000 fold more sensitive by QPCR than by growth on selective agar (Table 1). Following O.N. growth in enrichment, both assays were equally as sensitive.

For PHP oysters received nitrogen immersion, side by side comparison of standard MPN vs. QPCR-MPN showed excellent correlation (R^2=0.97 by Pearson's correlation co-efficient) and no significant differences between the two assays (Table 2). Results were comparable for untreated oysters and for PHP oysters at both 1 and 7 days post treatment.

Samples results were also examined side by side for both Nitrogen Immersion and Nitrogen Tunnel PHP treatments (Figure 1). In statistical comparison of this data, utilizing both JMP from SAS and Minitab, both one way ANOVA and Tukeys post hoc tests show no significant differences ($p < .05$) between detection methods; utilizing mCPC (presumptive positive) and VVAP (confirmed positive) known as the FDA-MPN, and QPCR-MPN. Field trials indicated that QPCR offered an improved confirmatory assay compared to the standard method, given that it reduced time and labor costs while still permitting detection of *V. vulnificus* without the risk of false positives.

Table 1. Detection of *V. vulnificus* in artificially inoculated APW enrichment

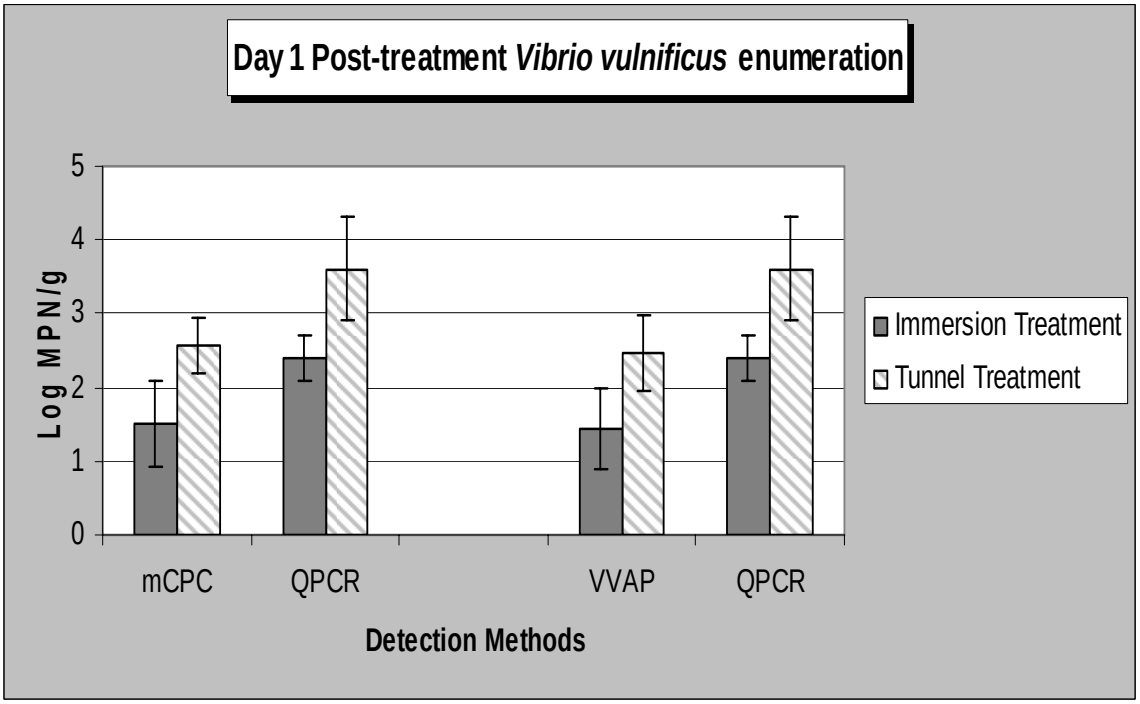
Inocula (log CFU/ml)	Pre-enrichment Positive APW (%)		Post-enrichment Positive APW (%)		Post- enrichment QPCR (C_t)	
	mCPC	QPCR Melt	mCPC	QPCR Melt	SYBR	TaqMan
0.01 g Oyster Homogenate:						
5.40	100	100	100	100	17.94±0.56	16.74±0.44
4.40	100	100	100	100	18.40±0.40	16.97±0.34
3.40	100	100	100	100	17.90±0.58	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) *V. vulnificus* inocula (logCFU/ml determined by plate count) for seeding of MPN enrichment of PHP oyster homogenates (0.01 or 0.10 g).
b) Detection of *V. vulnificus* in APW determined as percent (%) positive samples from three tubes based on either observation of *V. vulnificus* typical colonies on selective agar (mCPC) or on melt peak analysis (QPCR) for pre- and post-enrichment. Values are the mean of duplicate experiments with identical results.

Table 2. Comparison of standard MPN to QPCR-MPN Analysis of PHP Oyster Samples

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

a) Individual oyster lots (n=4) were heat abused by incubation at 26°C for 24h (Pre-PHP), followed by processing with ultralow freezing in liquid nitrogen and frozen storage at -10°C for 1 (PHP 1D) and 21 days (PHP 21D) following PHP.
b) For each lot, oysters (n=12) were sampled in triplicate, and average logMPN/g ± standard deviation determined by standard BAM method (FDA MPN) or by MPN using QCPR confirmation with SYBR Green I (QPCR-MPN), as described in text. Lots 1 to 3 were examined before and after PHP, and lot 4 was examined only at 21D after PHP.



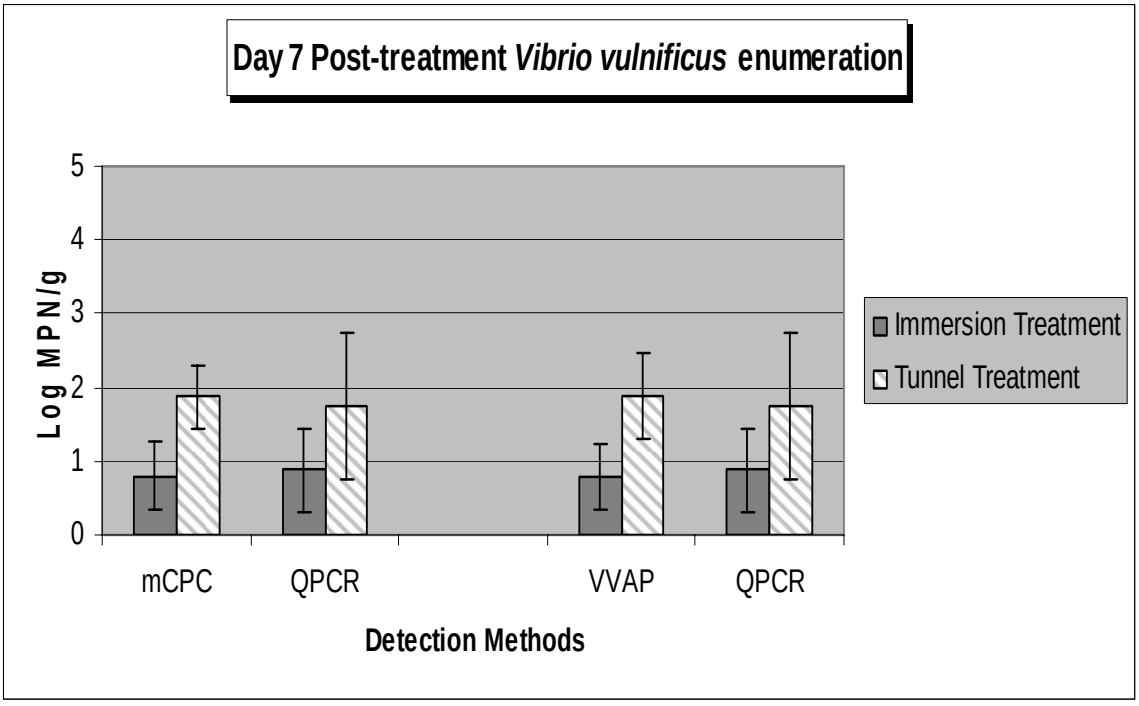


Figure 1. MPN's of temperature abused nitrogen treated samples. Comparison of detection methods, mCPC, VVAP and QPCR. Immersion treatment day 1 $p<0.09$, Immersion treatment day 7 $p<0.95$, Tunnel treatment day 1 $p<0.8$, and Tunnel treatment day 7 $p<0.95$.

Limit of Quantitation and Specificity

Wright et al., 2007, entitiled "Evaluation of Postharvest-Processed Oysters by Using PCR-Based Most-Probable-Number Enumeration of *Vibrio vulnificus* Bacteria", details the limits of quantitation and specificity. Seeding studies, utilizing known concentrations of *V. vulnificus* to spike oyster homogenates, were performed for a side by side comparison of mCPC selective media with QPCR results (Table 1). Although some loss of sensitivity was observed with the addition of 0.10 g oyster tissue at lower inocula, as compared to 0.01 g tissue, confirmation of positive samples in seeded homogenates prior to growth in APW still was about 100-fold more sensitive by QPCR melt peak than by recovery on mCPC. 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 0.10g and 0.01g homogenates (16). Thus, results confirmed that approximately one cell in the original inoculum could be detected by QPCR-MPN, in agreement with previous reports (1, 9, 10, 11). Positive and negative controls were included with each thermocycler run.

QPCR examination of DNA from *V. vulnificus* (n=25) and non-*V. vulnificus* (n=28) strains (Table 3) showed SYBR Green I detection was 100% sensitive to all *V. vulnificus* strains and species-specific for *V. vulnificus.* Results were confirmed by previously described TaqMan assay using identical primers with an additional TaqMan probe (2). C_t values (number of cycles required to reach threshold for detection) for SYBR Green I detection of *V. vulnificus* strains was comparable to TaqMan QPCR with mean C_t=16.48 ±0.79 and 16.61± 0.87, respectively. All *V. vulnificus* strains were positive by TaqMan assay while non-target species were all negative, including "false positives" stains (shown in bold in Table 1) described in the prior report. Although SYBR detected C_t values above threshold for non-target strains, detection only occurred after extended PCR cycling (mean number of cycles=34.86 ± 2.28), and is a consequence of artifactual signal (22). First derivative analysis of melting curves provides sensitive discrimination of nucleotide differences in the DNA sequence of amplicons (20, 22), and species-specific detection of PCR product by SYBR green I was confirmed by single melt peak with consistent values (mean= 88.02 ± 0.26) from *V. vulnificus* strains (Table 1). In contrast, melt peak values for non-target species averaged >22 standard deviations apart from the mean of positive controls. Melt peak analysis is recommended for confirmation of positive samples.

Table 1. Specificity and sensitivity of *V. vulnificus* QPCR detection with SYBR Green I and TaqMan detection.

T arget	QPCR[b]			Non-Target	QPCR		
Strains[a]:	**TaqMan (C_t)**	**SYBR (C_t)**	**Melt Peak**	**Strains:**	**TaqMan (C_t)**	**SYBR (C_t)**	**Melt peak**
Vibrio vulnificus				***Aeromonas***			
1009	16/46	16.15	88.29	***hydrophila* 7965**	0	34.77	70.76
MO6-24/O	16.14	16.17	88.10	*Escherichia coli*			
MLT365	ND[b]	18.29	88.15	JM109	0	37.42	82.68
6353	16.45	15.92	87.91	HB101	0	35.12	79.43
MLT367	17.21	17.6	88.42	*Listeria*			
CVD752	15.94	14.87	88.26	*monocytogenes*	0	36.11	78.15
345/T	15.60	16.36	87.77	*Pseudomonas*			
BO6312	17.56	16.44	88.12	*aeruginosa*	0	35.15	86.4
5C1326	ND	16.16	88.14	***shigelloides*14029**	0	35.12	76.69
NJMSA	15.91	15.65	87.83	*Salmonella enterica*			
UNCC1015	15.92	15.98	87.98	Cholerasius10708	0	36.04	77.97
CVD737	ND	16.13	87.93	Enterica10112	0	37.64	62.4
LC4	15.62	16.27	87.86	Enteridis13076	0	39.38	63.37
UNCC9	ND	16.32	88.02	Enteridis14050	0	38.99	62.66
85A667	ND	15.61	87.92	*V. cholerae*			
1015	16.16	15.87	88.13	JVY212	0	34.7	79.47
345/O	16.56	16.64	87.91	JVB 52	0	33.38	74.24
80363	15.72	16.09	88.78	JVY210	0	28.3	73.88
LC4/T	16.95	17.29	88.13	JVB 25	0	30.36	74.9

E4125	16.49	15.62	87.83	2076	0	35.06	79.59
2400112	18.2	17.34	88.08	A5	0	35.44	79.59
52785	ND	17.71	87.46	***V. alginolyticus***	0	33.18	77.16
EDL174	ND	16.5	87.91	*V. fisherii* ES114	0	38.44	63.17
MLT403	17.13	17.31	87.77	***V. fluvialis* 1959-2**	0	33.14	78.04
LL728	17.69	17.29	87.81	***V. furnissii* 1958-83**	0	34.35	78.76
				***V. hollisae* 89[a]7053**	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
Average:	16.61	16.48	88.02	Average:	0	34.81	75.90
	±0.87	±0.79	±0.26			±2.66	±6.75

a) Strains in bold were reported to be positive by prior study (16).
b) C_t values are shown for QPCR as described in text with melt peak analysis for SYBR Green I assay.

APPENDIX 1

DNA extraction:
For boiling lysis DNA extraction, 1 ml of APW IS centrifuged (15,000g) for 10 min. The supernatant is discarded and the pellet resuspended in 400 µl of phosphate-buffered saline (PBS). Sample is boiled for 10 min, and subsequently centrifuged for three minutes to remove particulates. Supernatants are stored at -20°C.

APPENDIX 2

Q-PCR method:
After cleaning work surfaces as described previously, a mastermix is prepared by conbining primers, sterile molecular grade PCR water, SYBR green I dye, and Cepheid© smartmix beads, according to manufacturer's specifications. Reagents are pippetted into Cepheid© tubes that have sterilized. The mastermix aliquot equals 23µl in volume for each sample. These tubes are then centrifuged for 5 seconds to ensure all reagents are in the bottom of the tube in the chamber. Primers were obtained from Geno-mechanix, Gainesville Florida. 1x SYBR green I dye (Invitrogen) is added to mastermix containing, vvhA primers (2, 16) and Cepheid© smartmix beads (1 bead per 2 reactions). DNA template (2 µl) or water are added to QPCR reactions for a total volume of 25 µl. The program utilized the following parameters, a hold for 2 min at 95°C followed by 40 cycles of 15 s at 95°C and 60°C for 1 min. Cycle threshold (*Ct*) values, the number of cycles required to reach threshold for detection, were compared to standard curve values to enumerate for SYBR green I detection of *V. vulnificus* strains. Analysis of melting peaks, curves representative of melting temperature, provide a sensitive discrimination of non target sequences in the DNA sequence of amplicons (12, 13), and species-specific detection of PCR product by SYBR green I was confirmed by single melt peaks in the target range.

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