Proposal Subject Real Time PCR Methods for Determining Levels of *V. parahaemolyticus* and *V. vulnificus*

Text of Proposal/ Requested Action

Real time PCR methods provide additional options to currently used methods for identification of *V. parahaemolyticus* and *V. vulnificus* in shellfish. The use of real time PCR could reduce the time and cost of analysis while providing more reliability for detection and identification of these organisms in the environment as well as in Post Harvest Processed (PHP) products. The following methods are submitted for consideration, under Procedure XVI, by the ISSC Laboratory Methods Review Committee for identification and characterization of suspect bacterial isolates and direct analysis of APW enrichments from MPN analysis.

Real time PCR assays for total V. vulnificus (SYBR green and taqman) developed at the University of Florida (2,3,4) and at the University of Alabama-Birmingham (7).

Real time multiplex PCR assays (taqman) for total and pathogenic *V. parahaemolyticus* developed at FDA GCSL (8) and at the University of Alabama-Birmingham (1,6,9).

Real time PCR offers rapid, quantitative analysis for detection of a number of food-borne pathogens. The University of Florida and the University of Alabama at Birmingham have developed real time PCR assays for detection of the *V. vulnificus* vvh gene (2,3,4,7). The proposed methods can be used either in a Taqman or SYBR green format and could be applied in an MPN format as an alternate confirmation tool for identification of bacterial isolates in the validation and verification of PHP oysters. There is also the potential to significantly reduce the

workload and time needed for analysis by using the PCR methods directly on APW enrichments, thus eliminating the need for streaking for isolation on selective media.

FDA has developed a multiplex real time PCR assay for *V. parahaemolyticus* species identification (*tlh*), virulence characterization (*tdh* and *trh*) and an internal amplification control to detect false negatives that could result from the presence of PCRinhibitors in the sample matrix (9). Currently the ISSC uses a DNA probe colony hybridization assay for quantifying total and pathogenic *V. parahaemolyticus* in oysters at harvest for the Interim Control Plan. The same method is also used for determining levels of *V. parahaemolyticus* after PHP. While this method can be completed in 24h, variations in signal strength sometimes complicate interpretations, especially at low colony numbers where this method is normally applied. Conversion of the *V. parahaemolyticus* procedure to an MPN format would also permit utilization of the same validation and verification procedures as used with *V. vulnificus*.

The University of Alabama at Birmingham has developed a method for the detection of *V. parahaemolyticus* in oysters using multiplexed real time PCR with taqman fluorescent probes (1,6,9). The current ISSC adopted procedure uses a colorimetric DNA probe colony hybridization assay for the detection of total and pathogenic *V. parahaemolyticus* targeting *tlh* and *tdh* genes. However, with this multiplex method, targeting additional genes, it is possible to achieve a comprehensive detection of all pathogenic forms of *V. parahaemolyticus* known to date in a single reaction tube by real-time Taqman-PCR method. The proposed method developed by the University of Alabama – Birmingham uses a multiplexed Taqman PCR-based detection of total (targeting *tlh* gene), pathogenic (targeting *tdh* and *trh* genes), and pandemic strains of *V. parahaemolyticus* O3:K6 serotype (targeting ORF8 gene) in a single reaction that could potentially be applied for routine monitoring of molluscan shellfish for this pathogen. This method appears to be specific and can be used for the detection of <10 cfu *V. parahaemolyticus* following overnight enrichment in T_1N_1 broth. Further, this method has the potential to confirm MPN enrichment method of detection of this pathogen by direct amplification of the targeted genes without further culture-based confirmation. The multiplexed PCR method of detection of total and pathogenic strains including the pandemic strain of *V. parahaemolyticus* is rapid; detection can be achieved in real-time amplification of the targeted genes; specific for the targeted pathogen; and sufficiently sensitive in enriched oyster homogenate to consider as an alternate method of

detection of this important pathogen.

Each of the proposed PCR methods were designed for use on the Cepheid Smart Cycler and would require some modifications to be used on other instruments. A review package including performance attributes will be provided for each method prior to the 2005 Conference.

References:

1. Bej, A.K., D.P. Patterson, C.W. Brasher, M.C.L. Vickery, D.D. Jones, C.A. Kaysner. 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh*, and *trh. Journal of Microbiological Methods* 36:215-225.
2. Campbell, M.S. and A.C. Wright. 2003. Real-time PCR analysis of *Vibrio vulnificus* from oysters. Appl.Enviro.Microbiol. 69:7137-7144.
3. Calero, A. G. (Wright, A. C., advisor). 2003. Application of molecular detection methods to most probable number (MPN) enumeration of *Vibrio vulnificus* in oysters. M.S. Thesis, University of Florida http://etd.fcla.edu/UF/UFE0002740/calero_a.pdf.
4. Harwood V. J., Gandhi, J. P., and Wright, A. C. 2004. Methods for Isolation and Confirmation of *Vibrio vulnificus* from Oysters and Environmental Sources: A Review. *J. Microbiol. Methods*. 59: 301-16.
5. Mead, P.S., L. Slutsker, V.Dietz., L.F. McGaig, J.S. Bresee, C. Shapiro, P.M. Griffin, and R.V. Tauxe. 1999. Food-related illness and death in the United States. Emerg.Infect.Dis. 5:607-625.
6. Myers, M., G. Panicker, A.K. Bej. 2003. Detection of newly emerged pandemic *Vibrio parahaemolyticus* O3:K6 pathogen in pure cultures and seeded Gulf waters using PCR. *Applied and Environmental Microbiology* 69:2194-2200.
7. Panicker, G., M.L. Myers, and A.K. Bej. 2004. Rapid detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water by real-time PCR. Appl.Enviro.Microbiol. 70:498-507.
8. Vickery, M.C.L., G.M. Blackstone, J.L. Nordstrom, and A. DePaola. 2003. Detection and quantification of total and potentially virulent *Vibrio parahaemolyticus* using a 4-channel multiplex real-time PCR targeting the *tl, tdh*, and *trh* genes and a novel PCR internal control. ASM Annual Meeting, Washington, DC, May 18-22, 2003.
9. Ward, L.N. and A. K. Bej. Detection of total and pathogenic *V. parahaemolyticus* in shellfish using multiplexed real-time pCR with Taqman fluorescent probes. (In preparation).

Public Health Significance

V. parahaemolyticus is the leading cause of bacterial gastroenteritis and *V. vulnificus* is the leading cause of death associated with seafood consumption in the US (5). ISSC has an ICP for Vp and has developed validation and verification of PHP for both organisms. Real time PCR is faster and more reliable than current methods but is not yet approved by ISSC. Approval of one or more of the proposed real time PCR methods would provide a faster and more reliable means of enumerating *V. vulnificus* and *V. parahaemolyticus* while offering an equivalent level of public health protection for consumers of raw molluscan shellfish.

Cost Information (if available)

None

Action by 2005 Laboratory Methods Review Committee

Recommended Proposal 05-107 be referred to the appropriate committtee as determined by the Conference Chairman, with further direction to the Executive Office to organize a meeting of the Laboratory Methods Committee within six (6) months of the conclusion of this Biennial Meeting.

Action by 2005 Task Force I

Recommended adoption of the Laboratory Methods Review Committee recommendation on Proposal 05-107.

Action by 2005 General Assembly	Adopted recommendation of 2005 Task Force I.
Action by USFDA	Concurred with Conference action.