Evaluation of a commercial multiplex QPCR assay for enumeration of *Vibrio* species in the Eastern oyster *Crassostrea virginica*

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Introduction

Vibrio vulnificus, *V. parahaemolyticus* and *V. cholerae* are the three *Vibrio* species that are most associated with human illness and are responsible for at least 75% of disease related to seafood-borne bacterial infections (2). These species are part of the natural microflora of the Eastern oyster, *Crassostrea virginica,* but their abundance and distribution are highly variable and dependent on key factors such as seasonal climate patterns, water temperature and salinity. Efforts to compare the distribution of the different Vibrios in the environment have been limited by lack of practical procedures for the simultaneous detection of all three species. A quantitative multiplex real-time PCR (QPCR) assay was recently developed for use with the Dupont BAX® Q7 system. The DuPont Qualicon BAX® Real Time *Vibrio* Test Kit allows rapid, simultaneous detection and enumeration of the three species in a most probable number (MPN) format using endpoint titration of samples from enrichment broth.

Results

Limits of Detection. Evaluation of the DuPont multiplex QPCR assay showed linear detection of the three *Vibrio* species for a range of 10^3 to 10^8 CFU/mL in alkaline peptone water (APW) enrichment broth that contained either 0.10 or 0.01 g of oyster homogenate (not shown). The limit of detection was also determined for QPCR vs. bacterial recovery on selective agars mCPC and TCBS, which are specific to recovery of *V. vulnificus* or able to support growth of all three *Vibrio* species, respectively. Prior to incubation, QPCR was initially more sensitive than selective agars for detecting different Vibrios in APW containing 0.1 g oyster homogenate, but results were identical for both assays after 24h incubation at 37°C. [Table 1].

TABLE 1. Detection of *Vibrio* species in artificially inoculated APW enrichment with 0.1 g oyster.

Inoculum	% Positive samples before APW enrichment			% Positive samples after APW enrichment		
(logCFU/mL)	mCPC	TCBS	QPCR	mCPC	TCBS	QPCR
V. vulnificus						
5.1	0	0	100	100	100	100
3.1	0	0	100	100	100	100
1.1	0	0	17	100	100	100
0.1	0	0	0	100	100	100
V. cholerae						
5.7	0	100	100	0	100	100
3.7	0	100	100	0	100	100
1.7	0	50	100	0	100	100
0.2	0	0	0	0	100	100
V. parahaemolyticus						
4.4	0	100	100	0	100	100
2.4	0	100	100	0	100	100
0.4	0	17	17	0	100	100
0.0	0	0	0	0	100	100
None 0	0	0	0	0	0	0

MPN Methods comparison. The MPN method (1) specified by the FDA Bacteriological Analytical Manual (BAM) was directly compared to QPCR-MPN analysis (4) using the BAX® assay for *V. parahaemolyticus* in oyster lots that were post-harvest processed (PHP).

The samples analyzed in these data included initial screening of unprocessed oysters (IS); temperature abused (TA) samples that were incubated overnight to increase levels of natural *Vibrios*; oysters treated by heat shock (HS); and oysters treated by blast freezing (BL) [Table 2]. Results showed that QPCR-MPN values were highly correlated with the values obtained by standard MPN that were confirmed by DNA probe (R^2=0.99). These data support that the use of QPCR-MPN analysis of PHP oysters as a highly sensitive quantitative assay that significantly reduces labor requirements and assay time in comparison to the traditional assay.

TABLE 2. Direct comparison of BAM and QPCR MPN in PHP oysters.

Oyster Lot	Log MPN/g	
	BAM MPN	QPCR MPN
25IS25	2.0±0.56	2.0±0.62
29IS	2.0±0.60	2.0±1.03
26TA	4.0±0.64	4.0±0.40
30TA	6.0±0.11	6.0±0.22
26HSD7	<3.0	<3.0
30HSD7	1.0±0.66	1.1±0.58
26BLD42	2.0±0.43	2.1±0.51

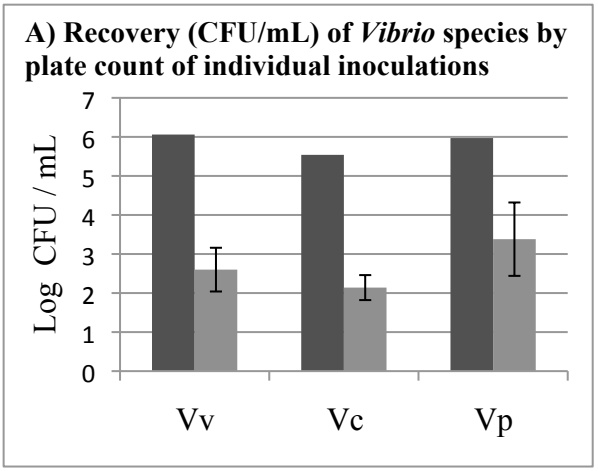
Field trials. The QPCR-MPN assay was evaluated from samples (n=3) collected in June 2010 from 3 sites in Apalachicola Bay, FL. Serial dilutions of oyster homogenates were inoculated into MPN enrichment tubes in triplicate, incubated overnight at 37°C, and subsequently streaked to selective media. Typical colonies were used to determine BAM MPN values for *V. vulnificus* (mCPC) and *V. parahaemolyticus* (CHROMagar™ Vibrio), but BAM MPN values were not determined (ND) for *V. cholerae*. Alternatively, enrichment cultures were evaluated by QPCR-MPN. Although DNA probe confirmation of the BAM MPN is pending, close agreement was observed between the BAM MPN and the QPCR-MPN assay for enumeration of *V. parahaemolyticus* and *V. vulnificus* (R^2=0.94 and 0.91, respectively) [Table 3].

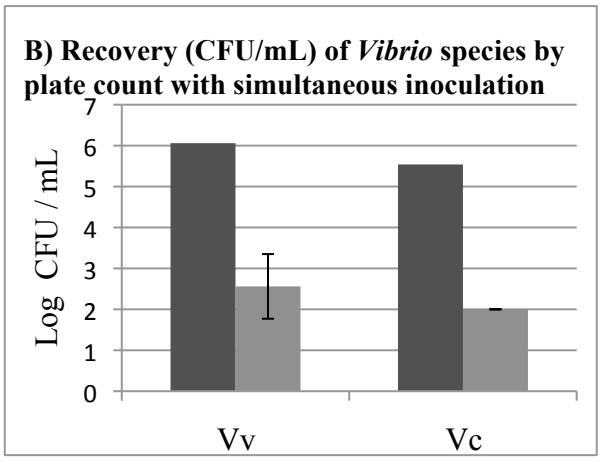
TABLE 3. Evaluation of BAM and QPCR MPN from oysters harvested at different salinities.

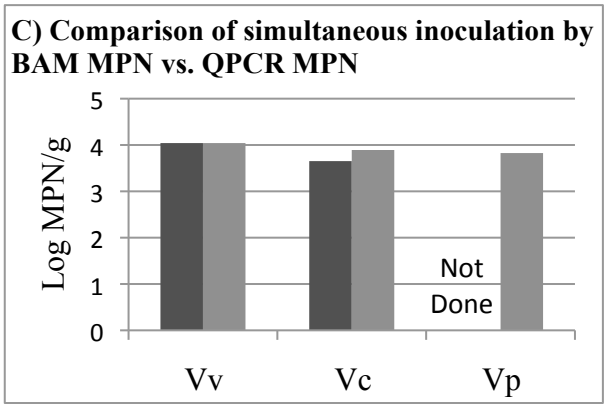
Site Name	Salinity (ppt)		Vv MPN/g		Vp MPN/g		Vc MPN/g	
	Surface	Bottom	BAM	QPCR	BAM	QPCR	BAM	QPCR
1. Ward's Lease	19.9	20.1	3.65	3.59	2.34	3.63	ND	0.30
2. Cat's Point	21.9	21.9	3.83	4.04	2.61	4.04	ND	0.48
3. Middle Site	9.3	16.8	4.64	4.57	3.92	2.75	ND	3.57

Recovery of Vibrios from live oysters. In order to determine a baseline for future evaluation of PHP methods to reduce Vibrios, live oysters were individually inoculated with the three *Vibri*o species using a previously described oyster model of infection at 16 ppt salinity and room temperature (3). The uptake of simultaneously inoculated *Vibrio* species was compared by plate count on selective media and by BAM MPN vs. QPCR-MPN. Although greater overall recovery of bacteria was observed from seawater, as compared to oyster meats, no significant differences were noted among individually inoculated species by plate count [Figure 1A]. Recovery by plate count also did not differ for *V. vulnificus* and *V. cholerae* when inoculated simultaneously [Figure 1B]. The MPN enrichment generally increased recovery from oysters compared to plate counts, as all species were $>10^3$ MPN/g by QPCR-MPN, and no significant differences were observed in enumeration using QPCR-MPN compared to standard assay [Figure 1C].

FIGURE 1. **Recovery of the different *Vibrio* species in live oysters.** Oysters were artificially inoculated with either A) individual *Vibrio* species or with B) simultaneous inoculation of all three species. Recovery was determined by plate count on mCPC or TCBS from seawater (dark grey) and oyster (light grey) samples. Comparison of the C) recovery from simultaneously inoculated oysters as determined by BAM MPN (dark grey) vs QPCR-MPN (light grey) assays was also evaluated.







Conclusions

QPCR-MPN was compared to the standard MPN for simultaneous detecton of three *Vibrio* species in *C. virginica*. Enumeration by QPCR-MPN showed no significant difference compared to BAM MPN in experimentally inoculated oysters and in field studies of natural populations. These data demostrate the potential of this assay for industrial application, due to the relatively quick assay time and the simplicity of the PCR protocol. QPCR-MPN was also used to calculate MPN/g uptake and recovery of the three *Vibrio* species in artificially seeded live oysters. Interestingly, Vp showed greater recovery than the other species under the conditions investigated. Future studies will investigate additional variables for competitive survival of the different species.

References

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