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Comparison of molecular detection methods for *Vibrio parahaemolyticus* and *Vibrio vulnificus*

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ABSTRACT

Pathogenic vibrios are a global concern for seafood safety and many molecular methods have been developed for their detection. This study compares several molecular methods for detection of total and pathogenic *Vibrio parahaemolyticus* and *Vibrio vulnificus*, in MPN enrichments from oysters and fish intestine samples. This study employed the DuPont Qualicon BAX® System Real-Time PCR assay for detection of *V. parahaemolyticus* and *V. vulnificus*. Multiplex real-time PCR detection of total (*tlh*+), *tdh*+, and *trh*+ *V. parahaemolyticus* was conducted on the Cepheid SmartCycler II. Total (*rpo*D) and *tdh*+ *V. parahaemolyticus* were also detected using LAMP. *V. vulnificus* detection was performed using real-time PCR methods developed for the SmartCycler and the AB 7500 Fast. Recommended template preparations were compared to BAX® lysis samples for suitability. There was no significant difference in detection of *V. parahaemolyticus* and *V. vulnificus* using the BAX® or SmartCycler assays. The AB assay showed no difference from other methods in detection of *V. vulnificus* unless boiled templates were utilized. There was a significant difference in detection of *tdh*+ *V. parahaemolyticus* between SmartCycler and LAMP assays unless the total (*tlh*+) *V. parahaemolyticus* gene target was omitted from the SmartCycler assay; a similar trend was observed for *trh*+ *V. parahaemolyticus*.

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1. Introduction

Vibrio spp. are the primary agents of bacteria-associated illness and mortality from seafood consumption in the U.S. and globally (Iwamoto et al., 2010). In the U.S., vibrio infections have been the leading cause of seafood-borne bacterial illness since 2001, with incidence increasing over the past decade (CDC, 2009). In 2008, 270 *Vibrio parahaemolyticus* and 85 *Vibrio vulnificus* cases were reported to the Centers for Disease Control and Prevention (CDC), approximately 65–70% of which were due to consumption of seafood (CDC, 2010). These infections resulted in 30 deaths from *V. vulnificus* and two from *V. parahaemolyticus*, similar to previous years, making these two species the leading cause of illness and mortality (CDC, 2010). The main vehicle for *V. parahaemolyticus* and *V. vulnificus* infections in the U.S. is consumption of raw molluscan shellfish (Anonymous, 2010; WHO/FAO, 2010). Additionally, the Food Aquaculture Organization and World Health Organization (FAO/WHO) *V. parahaemolyticus* risk assessment found that contamination of muscle tissue with intestinal contents during filleting and evisceration presented the greatest risk of *V. parahaemolyticus* infection from ingestion of raw finfish (FAO/WHO, 2011). Previous studies indicate the intestines of finfish can harbor high levels of *V. vulnificus*, making contamination of edible tissue a probable route of infection (DePaola et al., 1994).

Most regulatory entities around the globe rely on culture confirmation from food products to definitively identify the causative agent of food-borne illnesses. However, molecular detection methods such as conventional and real-time PCR, and loop-mediated isothermal amplification (LAMP) have become increasingly widespread in use as research and environmental survey tools. Pathogenicity of *V. parahaemolyticus* is strongly linked to production of the thermostable direct hemolysin (TDH) and/or the thermostable direct-related hemolysin (TRH), though other virulence factors are likely involved (Honda et al., 1988, 1990; Yeung and Boor, 2004). Therefore, many molecular detection assays have targeted the genes responsible for production of

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the *tdh* and *trh* hemolysins (Bej et al., 1999; Nemoto et al., 2009; Nordstrom et al., 2007; Tada et al., 1992; Yamazaki et al., 2010). For detection of total *V. parahaemolyticus*, a variety of molecular assays have been designed utilizing different targets such as the thermolabile hemolysin (*tlh*) and *tox*R genes, and the R72H fragment (Bej et al., 1999; Chen and Ge, 2010; Nordstrom et al., 2007; Yamazaki et al., 2008). Similarly, many molecular methods for detection of *V. vulnificus* are available, most targeting the *V. vulnificus* hemolysin (*vvh*A) or *tox*R genes (Campbell and Wright, 2003; Panicker and Bej, 2005; Ren et al., 2009; Takahashi et al., 2005). The BAX® System Real-Time PCR assay for *Vibrio cholerae*/*parahaemolyticus*/*vulnificus* detection is distinctive in that it does not utilize any of these targets for detection, so it provides a unique comparison of detection methods to this study. Gene targets selected for simultaneous detection of total *V. vulnificus* and total *V. parahaemolyticus* are proprietary, highly conserved housekeeping genes.

Although many molecular methods have been developed and applied across the globe, very little data exists on how these methods directly compare to one another. This type of comparison data is critical in evaluating the relevance of data generated using these different methods. The purpose of this study was to compare several detection methods for total and pathogenic *V. parahaemolyticus* and *V. vulnificus* in oyster tissue and fish intestines, two likely matrices for routes of infection. The methods utilized in the comparison were the BAX® System Real-Time PCR assay for *V. cholerae/parahaemolyticus/vulnificus*, (DuPont Qualicon, Wilmington, DE), the FDA real-time multiplex PCR for detection of total and pathogenic (*tdh*+, *trh*+) *V. parahaemolyticus* (Nordstrom et al., 2007), a loop-mediated isothermal amplification (LAMP) assay for total *V. parahaemolyticus* (Nemoto et al., 2011), a LAMP assay for *tdh*+ *V. parahaemolyticus* (Nemoto et al., 2009), the University of Florida real-time PCR for *V. vulnificus* (Campbell and Wright, 2003), and the National Institute of Health Sciences (Japan) real-time PCR for *V. vulnificus* (Takahashi et al., 2005). Although the BAX® Real-Time assay detects *V. cholerae* in addition to *V. parahaemolyticus* and *V. vulnificus*, we were unable to identify another molecular method for detection of total *V. cholerae*, therefore, no comparison was made.

2. Materials and methods

2.1. Sample processing

2.1.1. Oyster collection and processing

Oysters were collected by dredge near the mouth of Fowl River in Mobile Bay, Alabama and transported at ambient temperature to the FDA Gulf Coast Seafood Laboratory on Dauphin Island, Alabama. Fourteen oysters were held overnight (20–24 h) at each of three temperatures: 4 ± 1 °C, 25 ± 1 °C, and 35 ± 1 °C. Experiments were conducted during three independent collections in December 2008, April 2009, and June 2009. Oysters were scrubbed, shucked, and blended without dilution following APHA guidelines (APHA, 2001). A three-tube MPN was prepared as described in FDA's Bacteriological Analytical Manual (BAM) with slight modifications (DePaola and Kaysner, 2004). Briefly, three 10-g portions of homogenate were weighed into 90 g of APW (alkaline peptone water; 1% peptone, 1% NaCl, pH 8.5 ± 2). Three 1 g portions were weighed into 9 ml of APW. A 1:10 dilution of homogenate was made by weighing 1 g into 9 ml of PBS. Subsequent 10-fold dilutions were made into PBS, then 1 ml aliquots of each dilution were transferred to three tubes containing 9 ml of APW. All MPN tubes were incubated overnight (18–24 h) at 35 ± 1 °C. Aliquots from each turbid APW tube were removed and prepared for molecular analyses as described in Section 2.3.

2.1.2. Fish collection and processing

Finfish were collected using hook and line from a natural gas platform in the Gulf of Mexico and the mouth of Fowl River in September 2008, from Mobile Bay and Gulf of Mexico in October 2008, and from Fowl River in December 2008. Fish were placed on bagged ice and transported to the FDA Gulf Coast Seafood Laboratory. All fish were analyzed within 4 h of catch. Intestinal contents of fish were aseptically removed as previously described (DePaola et al., 1994) and placed into a sterile Pulsifier bag (Microbiology International, Frederick, MD). Intestinal contents were diluted 1:10 with PBS and mixed in a Pulsifier for 90 s. An MPN was set up by transferring three 1-ml portions to 9 ml APW. Subsequent dilutions were made in PBS (20 μl:180 μl) and used to inoculate APW in a microtiter plate (DePaola et al., 1994). The microtiter plate was incubated overnight (18–24 h) at 35 ± 1 °C. Aliquots from each turbid well were removed and prepared for molecular analyses as described in Section 2.3.

2.2. Sample preparation

2.2.1. BAX® System lysis sample preparation

BAX® System lysis samples were prepared according to manufacturer's recommended protocol (DuPont Qualicon, Wilmington, DE). Briefly, lysis solution was prepared by adding 150 μl of protease to 12 ml of lysis buffer, both of which are provided in the BAX® kit. This lysis solution was then divided into 200 μl aliquots, to which a 5 μl aliquot from each turbid MPN tube was added. Samples were incubated at 37 °C for 20 min, then heated at 95 °C for 10 min to inactivate protease activity. Samples were placed in a cold block to rapidly chill (at least 5 min).

2.2.2. Boiled sample preparation

Boiled samples were prepared as previously described (Blackstone et al., 2003; Nordstrom et al., 2007) by transferring 1 ml from each turbid APW tube into a microcentrifuge tube. Samples were heated at 100 °C for 10 min then placed on ice until cool. Samples were centrifuged at 13,000 × *g* for 2 min immediately prior to use, and the resulting supernatant was used for analysis.

2.2.3. Alkaline extract preparation

Alkaline extracted samples for testing by LAMP were prepared by transferring 50 μl from each turbid APW tube to a microcentrifuge tube. To each sample, 42 μl of 50 mM NaOH was added and heated at 100 °C for 5 min. Samples were placed on ice until cool. To each sample, 8 μl 1 M Tris–HCl, pH 7 was added and mixed gently. Samples were centrifuged at 2000 × *g* for 1 min prior to use, and resulting supernatant used for analysis.

2.3. Molecular analyses

2.3.1. BAX® System Real-Time PCR assay for V. cholerae/parahaemolyticus/vulnificus

For detection of *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*, samples were analyzed per manufacturer's recommended protocol (DuPont Qualicon). Only BAX® System lysis samples were used as template. Thirty μl of each sample was transferred using a multichannel pipettor to a PCR tube containing a BAX® System PCR Vibrio assay tablet. Tubes were sealed using provided optical flat caps. Samples were placed in the BAX® System Q7 cycler/detector and the Vibrio protocol was run.

2.3.2. Non-commercial Real-Time PCR assays

For detection of total and pathogenic (*tdh*+, *trh*+) *V. parahaemolyticus*, the FDA real-time PCR method reaction conditions and cycling parameters were as previously described