RAPID DETECTION OF TOTAL AND PATHOGENIC *VIBRIO PARAHAEMOLYTICUS* USING REAL-TIME PCR WITH TAQMAN® FLUORESCENT PROBES

Evan Markegard, Student Author
Marie Yeung, Research Advisor

ABSTRACT

Vibrio parahaemolyticus is found throughout the marine environment and is a major cause of foodborne illness around the world. Foodborne illnesses due to *V. parahaemolyticus* infections frequently trace back to the consumption of raw or undercooked seafood. The ability to detect *V. parahaemolyticus* in a rapid, sensitive, and specific manner is important to safeguard our food supply and prevent consumption of tainted shellfish. The objective of this study was to optimize a

detection assay for *V. parahaemolyticus,* with a focus on the pathogenic strains, based on real-time polymerase chain reaction (PCR) technology using pure cultures. Primers and TaqMan® fluorescent probes were custom designed to anneal to both the species specific thermolabile hemolysin *(tlh)* gene and the virulence marker thermostable direct hemolysin *(tdh)* gene to detect for the presence of total and pathogenic *V. parahaemolyticus* strains, respectively. Different strains (*n*=14) of *V. parahaemolyticus* and other *Vibrio* species were tested in the real-time PCR assay. Conventional PCR results obtained in a previous study were used as the basis of comparison for determining sensitivity and specificity. The detection of *tlh* had 100% sensitivity (12/12) and specificity (2/2), whereas the *tdh* gene had 100% sensitivity (6/6) and 88% specificity (7/8). In addition, a standard curve using threshold cycle (C_T) values was constructed for both genes and had R_2 values of 0.98 and 0.99 for *tlh* and *tdh*, respectively. The limit of detection was 9.16 pg (for *tlh*) and 6.13 pg (for *tdh*) of purified *V. parahaemolyticus* DNA, corresponding to 2.2×10^3 and 1.5×10^3 cfu/ml, respectively. A rapid molecular detection method using real-time PCR was successfully developed and optimized for the detection of *tlh* and *tdh* in *V. parahaemolyticus*.

INTRODUCTION

Vibrio parahaemolyticus is a gram-negative bacterium commonly found in estuarine and temperate coastal marine waters throughout the world (Ward and Bej, 2006). The organism can be found both free living or as commensals with reservoir hosts including fish and shellfish (Ward and Bej, 2006). Humans are susceptible hosts who can be infected through the consumption of raw or undercooked shellfish, particularly oysters (Yeung and Boor, 2004). Coastal countries have a greater risk of infection and outbreaks have occurred in North America, Southeast Asia, Japan, Russia, Spain, Taiwan and India (Ward and Bej, 2006). Outbreaks in the United States have occurred in the Pacific Northwest, Gulf, and Atlantic coastal regions.

V. parahaemolyticus is often associated with seafood-borne illnesses. Within 48 hours of ingesting the pathogenic strains, gastroenteritis symptoms appear

which include hyper secretion, gastroenteritis, nausea, vomiting, chills, fever, watery diarrhea, and sometimes abdominal cramping (Yeung and Boor, 2004). Gastroenteritis caused by *V. parahaemolyticus* is a self-limiting infection and most infected patients will recover without treatment (ProMed-mail 2007). In more severe cases, however, fluid replacement therapy or antibiotics such as tetracycline or doxycycline may be administered to shorten recovery time (Park et al., 2004). The mortality rate is approximately 1% (Yeung and Boor, 2004).

Cooking seafood properly could drastically reduce the risk of contracting the *V. parahaemolyticus* infections as they are sensitive to heat treatment (ProMED-mail 2007). Nevertheless, the increasing popularity of consuming raw or undercooked oysters, coupled with warmer global water temperatures that are induce for the growth of *V. parahaemolyticus*, have partly led to the re-emergence of this food pathogen (Figure 1) (CDC 2007).

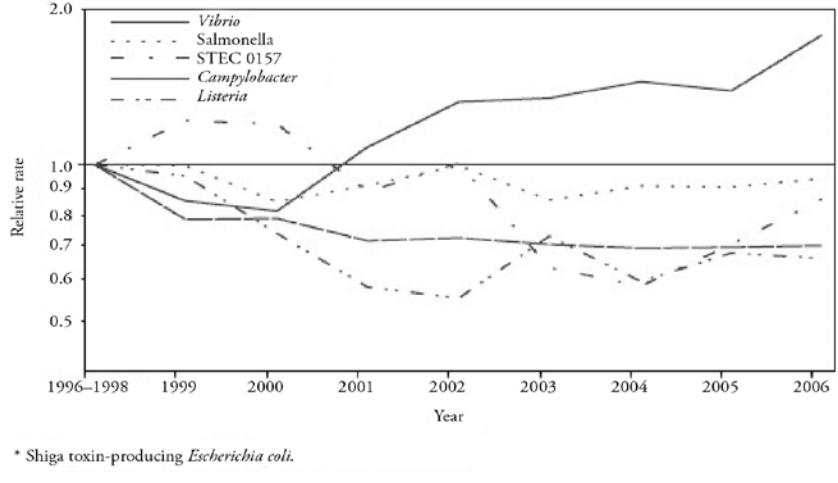

Figure 1. Among common foodborne pathogens in the United States, incidence of *Vibrio* (mostly *V. parahaemolyticus*) associated infection shows an upward trend (reprint from CDC 2007). The relative ratio is determined using the average number of illnesses in 1996-1998 as the denominator.

Ultimately, to prevent *V. parahaemolyticus* infections, harvested oysters should be screened for the presence of *V. parahaemolyticus* strains, particularly for those that have a pathogenic potential, prior to reaching the market place.

To this end, the objective of this study was to optimize a detection assay for *V. parahaemolyticus*, with a focus on the pathogenic strains, based on real-time polymerase chain reaction (PCR) technology using pure cultures.

MATERIALS AND METHODS

To adopt real-time PCR, primers and probes were essential. New primers and TaqMan® fluorescent probes were designed to detect for the presence of *tlh* (indicative of non-pathogenic and pathogenic *V. parahaemolyticus*) and *tdh* (indicative of pathogenic strains) using Primer Express 2.0 and PrimerQuest. The sequence and other relevant properties are shown in Table 1. BLAST homology search against the NCBI GenBank database was done to verify the specificity of primers and probes. Primers and probes were supplied from Applied Biosystems Inc (Foster City, CA). DNA of *Vibrio* strains was extracted using a Qiagen Gram Negative Bacteria Purification Kit (Qiagen Inc, Valencia, CA). DNA was quantified using Qubit Quanti-iT DNA Broad Range Kit (Invitrogen Corp, Carlsbad, CA) and confirmed using Quant-iT PicoGreen dsDNA Kit (Invitrogen Corp). Final primer and probe concentrations were 0.4 µM and 0.2 µM respectively. All assays included a negative control made up of all reagents except template DNA was replaced with PCR grade H_2O.

An Applied Biosystems Real-Time PCR 7300 System was used. The cycling program consisted a 15 s denature step at 95°C, 20 s annealing step at 57°C (*tdh*) or 58°C (*tlh*), and a 30 s extension step at 60°C. After 35 cycles, threshold cycle values were determined according to the build-in computer software program. Standard curves were constructed by first quantifying DNA template constituting DNA extracts of cell suspension of *V. parahaemolyticus* FSL-Y1-005 and FSL-Y1-013, then conducting real-time PCR on 10-fold serial dilutions of these extracts. The bacterial cell number in the cell suspension was also determined concurrently by standard plate count on tryptic soy agar supplemented with 2% NaCl.

Table 1. Real-Time PCR primer and probe target sets for the *tlh* and *tdh* genes. Each set was custom designed using consensus sequences from NCBI BLAST and Primer Express 2.0. The melting temperature, GC content, length, and specific fluorescent dyes and quenchers were all considered when designing the primers and probes.

Target Gene	Oligo-nucleotide	Sequence (5'-3')	Tm (°C)	% GC	Length (nt)	Dye/ Quencher
tlh	Forward Primer	AAAGCGGATTATGCA-GAAGCACTG	63	46	24	
	Reverse Primer	CGATCTCTTCTTGTGTT-GAGTACTTAAACTG	61	39	31	
	Probe	TGTTGATGACACTGCCA-GATGCGACGA	72	52	27	6FAM/ TAMRA
tdh	Forward Primer	GTAAAGGTCTCTGACTTTT-GGACAAACC	62	43	28	
	Reverse Primer	AGTCATGTAGGATGTCAGC-CATTTAGTAC	60	41	29	
	Probe	CCGTACAAAGATGTTTATG-GTCAATCAGTATTCACAACG	71	38	39	VIC/ TAMRA

V. parahaemolyticus isolates were obtained from the US Food and Drug Administration. Molecular characterization on these isolates has been conducted in our previous study that showed that these isolates have different pulsotype (Yeung et al., 2002). Other characteristics of these isolates are shown in Table 2. *V. alginolyticus*, and *V. vulnificus* were obtained from Hardy Diagnostic (Santa Maria, CA).

RESULTS

Real-time PCR on *tlh* and *tdh* was conducted on 12 *V. parahaemolyticus* strains, 1 *V. alginolyticus* and 1 *V. vulnificus* strains. As expected, *V. alginolyticus* and *V. vulnificus* were negative for *tlh*- and *tdh*-PCR. All *V. parahaemolyticus* strains were positive for *tlh*. These results confirmed the primers and probes were specific for *V. parahaemolyticus*.

Table 2. Real-time and conventional PCR results from 14 different confirmed *Vibrio* isolates (actual quantitative C_T values not shown for clarity). All real-time PCR results were replicated at least once. Proper controls were used in all experiments. Conventional PCR results were determined previously by our lab.

***Vibrio* strains**	**Serotype**	**Source of isolation**	***tlh*-PCR**		***tdh*-PCR**	
			Conventional[2]	**Real-Time**	**Conventional**	**Real-Time**
V. alginolyticus						
ATCC 17749	NA[1]		ND[3]	-	ND	ND
V. vulnificus						
ATCC 29307	NA		ND	-	ND	ND
V. parahaemolyticus						
FSL-Y1-001	O8	Food (clam)	+	+	-	-
FSL-Y1-002	O4:K8	Food (oyster)	+	+	+	+
FSL-Y1-005	O1	Food (oyster)	+	+	-	-
FSL-Y1-006	O5	Food (oyster)	+	+	-	-
FSL-Y1-010	O5:K15	Clinical	+	+	+	+
FSL-Y1-012	O4:K12	Clinical	+	+	-	-
FSL-Y1-013	O6:K18	Clinical	+	+	-	+
FSL-Y1-014	O4:K68	Clinical	+	+	+	+
FSL-Y1-016	O3:K6	Clinical	+	+	+	+
FSL-Y1-017	O3:K6	Clinical	+	+	+	+
FSL-Y1-021	O3:K6	Clinical	+	+	+	+
FSL-Y1-022	O3:K6	Clinical	+	+	-	-

[1] Not applicable

[2] Results from a previous study (Yeung et al., 2002)

[3] Not determined

The results for different *V. parahaemolyticus* strains were compared against previously published conventional PCR results (Table 2) to determine the sensitivity and specificity (Table 3). The values shown in Table 3 indicate false nega-

tive (sensitivity) and false positive (specificity) rates. Sensitivity and specificity for both genes was 100% except for specificity of *tdh* which was 88%. Specifically, *tdh* was detected in FSL-Y1-013 by real-time PCR but not by conventional PCR. We speculate that the primers used in real-time PCR may be more specific than those used in conventional PCR.

Table 3. Sensitivity and specificity of detecting *tlh* and *tdh* using real-time PCR as compared to using conventional PCR.

Gene	Sensitivity	Specificity
tlh	12/12 = 100%	2/2 = 100%
tdh	6/6 = 100%	7/8 = 88%

To determine the number of cells initially present in the reaction, a standard curve was constructed to correlate the threshold cycle (C_T) value with varying concentration of DNA (and thereby cell number). C_T is the number of cycle required to generate sufficient fluorescent signal to reach a pre-defined threshold level. The lower the amount of DNA (or cell) in the sample, the higher the C_T value. Figure 2 shows results from a representative standard curve experiment.

The limit of detection of *tlh* and *tdh* were 9.16 and 6.13 pg of DNA, respectively. These amounts of DNA corresponded to 2.2×10^3 and 1.5×10^3 cfu/mL, respectively.

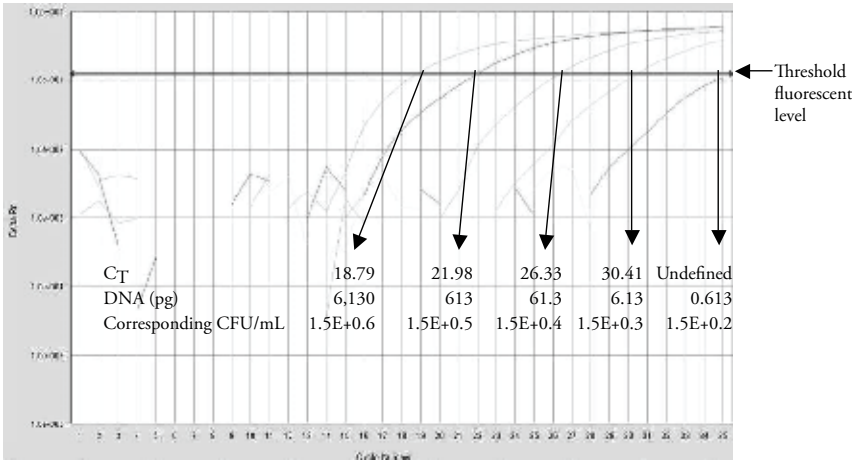

Figure 2. A standard curve using the log of threshold cycle (C_T) values was constructed for *tdh*. The negative control has no amplification. The *tdh* gene had a R^2 value from the standard curve of 0.99.

CONCLUSIONS

A rapid molecular detection method using real-time PCR was successfully developed and optimized for the detection of two *V. parahaemolyticus* genes, *tlh* and *tdh*. With the optimized real-time PCR assay, our next step is to determine its sensitivity and specificity using food samples. Future research directions could include primers and probes designed for other pathogenic markers besides *tdh*, such as the Type III Secretion System (Park et al., 2004) to enhance the utility of this approach. Having the ability to provide timely and accurate results on the prevalence of pathogen is critical to maintain and improve the safety of our food supply.

WORKS CITED

Centers for Disease Control and Prevention. 2007. Preliminary FoodNet Data on the Incidence of Infection with Pathogens Transmitted Commonly Through Food --- 10 States, 2006. Morb Mortal Wkly Rep. 56:336-339.

Honda, T., Iida T., Akeda Y., Kodama T. 2008. Sixty Years of *Vibrio parahaemolyticus* Research. Microbe. 3:462-466.

Mackay, I., Arden, K., Nitsche, A. 2002. Survey and Summary: Real-time PCR in Virology. Nucleic Acids Research 30:1292-1305.

Park, K., Ono, T., Rokuda, M., Jang, M., Okada, K., Iida, T., Honda, T. 2004. Functional Characterization of Two Type III Secretion Systems of *Vibrio parahaemolyticus*. Infection and Immunity 72:6659-6665.

ProMED-mail. 2007. *Vibrio parahaemolyticus*, oysters - USA (WA): int'l aler. ProMED-mail 2007; 13 Aug: 20070813.2637. <http://www.promedmail.org>. Accessed 21 Jan 2008.

Ward, L., Bej, A. 2006. Detection of *Vibrio parahaemolyticus* in Shellfish by Use of Multiplexed Real-time PCR with TaqMan Fluorescent Probes. Applied and Environmental Microbiology, 72:2031-2042.

Yeung PS, Hayes MC, DePaola A, Kaysner CA, Kornstein L, Boor KJ. 2002. Comparative phenotypic, molecular, and virulence characterization of *Vibrio parahaemolyticus* O3:K6 isolates. Applied and Environmental Microbiology 68:2901-2909.

Yeung, M., Boor, K. 2004. Epidemiology, Pathogenesis, and Prevention of Foodborne *Vibrio parahaemolyticus* Infections. Foodborne Pathogens and Disease. 1:74-88.