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Short Communication

Rapid Detection of *Vibrio harveyi* in Seawater by Real-Time PCR

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Vibrio harveyi cause mass mortalities of cultured marine fish. To address the ecology of *V. harveyi* in aquaculture, intensive monitoring is needed. We first optimized a quantitative real-time PCR method to determine *V. harveyi* abundance. The designed TaqMan probe and primers based on the 16S rRNA gene were specific at 68°C of annealing and extension. Furthermore, the method using a chelating resin method was able to enumerate 1.7×10^2 CFU/ml in breeding seawater at an abalone farm. This method represents a good tool for monitoring the ecology of *V. harveyi* in marine environments within 5 h.

Key words: quantitative real-time PCR, *Vibrio harveyi*, rapid detection

Vibrio harveyi has been recently recognized as a serious pathogen of various marine fish and invertebrates all over the world[1]. Abalone farms in Japan also encounter serious economic losses caused by *V. harveyi* infection[10]. In one incident, an infection caused sudden death of 60,000 animals within a couple of days without any predictive signs[10]. However, the *V. harveyi* dynamics that caused such a quick change of physiological imbalance between the bacteria and host animals remained to be clarified.

Culture-dependent detection such as a colony PCR of *V. harveyi* involves 3-days of replica plating, PCR, and electrophoresis[4]. Recently, MPN-PCR methods for enumerating *V. harveyi* by targeting *gyrB* genes had a high detection limit (15 cells/ml), but still required a whole day including an overnight enrichment step[12]. Accordingly, these culture methods have not enough power for daily monitoring of sudden abalone death triggered by *V. harveyi*.

The development of real-time PCR methods has enabled us to quantify the abundance of nucleic acids with rapidity, simplicity, and high accuracy[8]. No real-time PCR methodology to quantify *V. harveyi* has been reported yet because there are some extremely similar species such as *V. rotiferianus* and *V. campbellii*[5–7,13]. In *Vibrionaceae*, the inter-specific discriminatory power of 16S rRNA gene (99.6%) was lower than other protein coding genes such as *gyrB* (88.5%)[11]. However, some housekeeping genes such as *gapA*, *gyrB*, and *recA* genes, unpredictably, showed low discriminatory power for identification between *V. harveyi* and *V. campbellii*[13,14]. The *V. harveyi* specific region of 16S rRNA gene (*E. coli* positions 175–194) was recently found[4]. Therefore, since sufficient 16S rRNA gene sequences are present in public databases, the gene is still the best gene for the development of a species-specific detection assay. To increase the specificity of the quantitative real-time PCR, we selected 16S rRNA gene as the target gene.

Filtration is generally used in DNA preparation from several liters of water samples. However, this step might be critical for rapidity, and lead to low sensitivity (10^2–10^3 cells/ml) due to loss of cells during recovery from the filter surface and/or during the rigorous purification steps[2]. A filtration-DNA extraction step is unlikely to be suitable for daily monitoring of the bacterial abundance in an intensive aquaculture facility. Therefore, we attempted to develop a simple method from a small volume (1 ml) of water sample within one hour. Once a combined detection system for *V. harveyi* based on real-time PCR with simple DNA preparation method has been established, the method will be useful to understand how quickly *V. harveyi* abundance changes, allowing us to predict future outbreaks of mass mortalities by *V. harveyi* infection, and to establish strategies for minimizing the death of marine fish and shellfish in aquaculture facilities.

A region of the 16S rRNA gene corresponding to *E. coli* positions (AE014075) 175–194 is known as a region which is highly conserved in *V. harveyi* strains[4]. The region used was a part of the sequence of RTVHA TaqMan probe (5'-CCGCATAATACCTACGGGTCAAAGAGGG-3'). The probe was labeled with FAM at the 5' end and TAMRA at the 3' end. Candidate primer sets were searched based on the above described TaqMan probe at the center by Primer Express Software v2.0 (Applied Biosystems, Foster City, USA). The primer set used was as follows: RTVHAF primer (5'-CGAGCGGAAACGAGTTATCTG-3') and RTVHAR primer (5'-CTCACCAACTAGCTAATCCCACCTA-3'). Mismatches against probe and primer sequences were evaluated by the Ribosomal Database Project II[9]. Twenty species that had less than four nucleotide mismatches against designed probe and primers sequences were selected to evaluate the specificity. Genomic DNA from each strain was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, USA) and suspended in TE buffer (10 mM Tris-1 mM EDTA; pH 8.0). The DNA concentration was measured in a spectrophotometer at a wavelength of 260 nm.

A subsample (1 μl) of genomic DNA (25 ng/μl) was added to PCR reaction mixtures (total 25 μl), which contained each

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primer at concentrations of 600 nM, 250 nM TaqMan probe, and TaqMan Core Reagents (1×TaqMan Buffer A, 5 mM $MgCl_2$, 200 mM dATP, dCTP, dGTP, 400 mM dUTP, 0.25 U AmpEraseUNG, 0.625 U AmpliTaq Gold DNA Polymerase) (Applied Biosystems, Foster City, USA). The reaction was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, USA). PCR cycles consisted of incubation for 2 min at 50°C and for 10 min at 95°C, followed by 45 cycles of denaturation for 15 s at 95°C and annealing-extension for 1 min at 60, 62, 64, 66, or 68°C. The setting of baseline and threshold line was analyzed using the ABI PRISM 7000 SDS Software. The baseline was set based on a reaction with no template DNA, and the threshold line was set at around the end of an exponential amplification. For evaluation of probe and primer specificity, a *Ct* value within 45 was considered to be positive.

The sensitivity of real-time PCR was examined using purified genomic DNA extracted from a culture of *V. harveyi* strain S20. Purified DNA from *V. harveyi* was diluted from 25 ng to 25 fg. The minimum DNA amount where the *Ct* value was within 45 for all triplicate samples was considered as the detection limit.

In the case of the co-existence of *V. harveyi* and related species, the influence of other vibrio DNA on the quantitation of *V. harveyi* was evaluated with real-time PCR. *V. rotiferianus* was selected as the related species having the most similar sequences of probe (2 mismatches) and primers (0 mismatch). DNA mixtures were composed of various combinations of *V. harveyi* (12.5 ng/μl, 12.5 pg/μl and 1.25 pg/μl) and *V. rotiferianus* (125 ng/μl to 125 fg/μl) genomic DNAs. A subsample (1 μl) of the DNA mixture was added to the PCR mixture. A reaction containing *V. harveyi* DNA alone (25 ng to 25 fg) was used as a positive control.

The effect of DNA preparation methods on the sensitivity of the real-time PCR was examined. *V. harveyi* S20 was seeded in autoclaved natural seawater (10^6 to 10^0 CFU/ml). 1 ml of each sample was then centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was carefully discarded. These bacterial pellets were used for the following three DNA extraction methods. The boiling method was performed as follows. The above mentioned cell pellets were suspended in 200 μl sterile distilled water and vortexed for 20 seconds. The cell suspension was boiled at 100°C for 10 min and vortexed for 10 seconds. The chelating resin method was performed according to Walsh *et al.*[15]. In brief, centrifuged cell pellets were suspended in 200 μl sterile 5% chelating resin (Sigma, St. Louis, USA) and vortexed for 20 seconds, followed by incubation at 56°C for 20 min and subsequent vortex for 10 seconds. The suspension was incubated at 100°C for 10 min, then vortexed for 10 seconds. DNA preparation with the DNA Purification kit was performed according to the manufacturer's instructions (Promega, Madison, USA) and purified DNA was suspended in 200 μl TE buffer. The sample was stored at −20°C. Each DNA sample was centrifuged (14,000 rpm, 5 min) and a 3 μl aliquot of the supernatant was used for real-time PCR. The minimum viable counts (CFU/ml) where the *Ct* value was within 45 for all triplicate samples were considered as the detection limit.

Cells of *V. harveyi* in the natural environment can show a variety of physiological states, ex. stressed or starved. Therefore, the effect of starvation on the quantitation of *V. harveyi* cells was evaluated in microcosms using artificial seawater. *V. harveyi* S20 was grown in 100 ml of ZoBell 2216E broth at 130 rpm until the OD620 reached 1.0. The culture was harvested by centrifugation at 5,000 rpm for 10 min, and then the cells were washed twice in sterilized Tris-buffered 75% artificial seawater (TB-ASW) (4 mM Tris-HCl, pH 8.0) to minimize carryover of the medium. A total of 20 ml cell suspension was added to a flask containing 180 ml of TB-ASW, then incubated in the dark at 20°C without shaking. After 0, 7, and 14 days, the cell suspension was taken, diluted 10-fold in autoclaved natural seawater, and then centrifuged for 10 min at 14,000 rpm. The cell pellet was processed with a DNA extraction method based on the chelating resin. Each sample was subjected to viable CFU counts and 4',6-diamidino-2-phenylindole (DAPI) direct counts. Viable CFU counts were determined by using ZoBell 2216E agar plate count for 2 days at 20°C. DAPI stained cells were enumerated by epifluorescence microscopy through a No. 1 filter (excitation 365 nm, emission 397 nm) (Axioskope, Zeiss, Germany). The detection limit was evaluated by minimum direct counts (cells/ml) where the *Ct* value was within 45 for all triplicate samples.

Seawater samples were collected in August and September 2005; coastal seawater was taken at a point 600 m distant from the abalone farm, and supplying and breeding seawater samples were taken at the farm. A mass mortality of cultured abalone occurred on 23 August 2005. They were sampled in sterilized bottles and transported to our laboratory on ice. In each sample, three subsamples (1 ml) were used for DNA preparation with the chelating resin method. These samples were stored at −20°C. A portion (3 μl) of supernatant was used for quantitative real-time PCR. A subsample (0.1 ml) of seawater was plated onto ZoBell2216E agar and incubated at 20°C for 7 days. Given that the samples had bacterial numbers at below detectable levels by the spread plate method, volumes of 1 to 200 ml seawater were filtered through a 0.2-μm Nuclepore filter, and the filters were placed onto TCBS agar and incubated at 20°C for 2 days. Individual colonies were grown on ZoBell2216E agar and TCBS agar at 20°C for 2 days, and sucrose-positive colonies were selected. The colonies were identified by the *V. harveyi*-specific colony PCR method[4].

The real-time PCR was optimized by varying annealing and extension temperatures (60–68°C). As the RTVHA probe had less than four nucleotide mismatches against other species (Table 1), the annealing temperature needed to be increased to 68°C to enhance the specificity of the probe. Real-time PCR with the 68°C annealing-extension step was able to detect all 19 strains of *V. harveyi* isolated from various sources out of 20 other *Vibrio* species. *Ct* values for *V. harveyi* strains ranged from 17.22 to 20.83 (Table 1).

Using purified genomic DNA from *V. harveyi* S20, the minimum detection limit was 1.25 pg. The determination coefficient (R^2) was 0.999, and a slope of −3.61 was obtained. The efficiency of the curve was 89.2%.

The effect of the co-occurrence of *V. rotiferianus* DNA on the quantification of *V. harveyi* by real-time PCR was tested. In the case of the reaction mixture containing equal amounts of genomic DNAs (12.5 ng, 12.5 pg, and 1.25 pg) of *V. har-*

Table 1. Specificity of the *Vibrio harveyi* species-specific real-time PCR at 68°C of annealing-extension temperature

Species	Strains	GenBank accession	RTVHA probe mismatch	*Ct*
V. harveyi	S20	AY750577	0	18.25
	S35	AY750578	0	17.37
	LMG7890	X74693	0	19.06
	LMG19643	AJ312382	0	17.32
	LMG19714	AJ312383	0	18.26
	LMG4044^T	AY750576	1	17.78
	NCIMB1280^T	AY750575	1	18.28
	913BZ62	—	—	17.73
	913SDZ20	—	—	20.05
	99WT11	—	—	17.22
	720WT44	—	—	17.78
	89SF200-3	—	—	17.30
	823BAZ5	—	—	20.83
	823DZ6	—	—	18.50
	823WBZ7	—	—	18.26
	818ODEZ12	—	—	18.42
	818ODDZ2	—	—	19.11
	823TEZ2	—	—	19.66
	823TDZ13	—	—	18.20
V. campbellii	LMG11216^T	X74692	2	>45
		AY035896	3	
		X56575	3	
V. chagasii	LMG21353^T	AJ316199	2	>45
V. lentus	LMG21034^T	AJ278881	2	>45
V. proteolyticus	LMG3772^T	X56579	2	>45
		X74723	3	
V. rotiferianus	LMG21460^T	AJ316187	2	>45
V. alginolyticus	LMG4409^T	X74690	3	>45
		X74691	3	
		X56576	4	
V. brasiliensis	LMG20546^T	AJ316172	3	>45
V. coralliilyticus	LMG20984^T	AJ440005	3	>45
V. mediterranei	LMG11258^T	X74710	3	>45
	LMG19703	AF007115	3	>45
V. natriegens	LMG10935^T	X74714	3	>45
V. nereis	LMG3895^T	X74716	3	>45
V. neptunius	LMG20536^T	AJ316171	3	>45
V. nigripulchritudo	LMG3896^T	X74717	3	>45
V. orientalis	LMG7897^T	X74719	3	>45
V. parahaemolyticus	LMG2850^T	X74720	3	>45
		M59161	4	
		X56580	5	
V. pelagius	ATCC25916^T	X74722	3	>45
V. tubiashii	LMG10936^T	X74725	3	>45
V. xuii	LMG21346^T	AJ316181	3	>45
V. agarivorans	LMG21448^T	AJ310647	4	>45

Table 2. Effect of *Vibrio rotiferianus* DNAs on quantitation of *Vibrio harveyi* by real-time PCR

Amt. of *V. harveyi* DNA	Amt. of *V. rotiferianus* DNA	*Ct*[a]	Estimated amt. of *V. harveyi* DNA[b]	Percentage (%)
12.5 ng	125 ng	25.76±2.40	135 pg	1
	12.5 ng	19.36±0.34	8.3 ng	66
	1.25 ng	18.60±0.16	13.5 ng	108
	0	18.52±0.24	—	—
12.5 pg	125 pg	37.64±1.51	65 fg	0.5
	12.5 pg	31.35±0.66	3.7 pg	30
	1.25 pg	29.33±0.27	13.5 pg	108
	0	28.76±0.27	—	—
1.25 pg	12.5 pg	>45	—	—
	1.25 pg	33.81±0.32	759 fg	61
	125 fg	32.76±0.29	1.51 pg	121
	0	32.56±0.35	—	—
0	25 ng	>45	—	—
0	0	>45	—	—

[a] Practical *Ct*. The data are shown by triplicate.
[b] Estimated amount of DNA by standard curve. y=−3.58x+40.55; x=log (DNA amount), y=(Practical *Ct*)

veyi and *V. rotiferianus*, estimated amounts of *V. harveyi* were estimated as 66%, 30%, and 61% of the initial amounts, respectively (Table 2). When *V. rotiferianus* DNA included one-tenth amounts of *V. harveyi* DNA, amounts of *V. harveyi* were estimated as 108%, 108%, and 121% in the reaction containing 12.5 ng, 12.5 pg and 1.25 pg of *V. harveyi* DNA, respectively. However, in the reaction with amounts of *V. rotiferianus* DNA 10 times higher than that of *V. harveyi* DNA, *V. harveyi* were out of range from less than 1% of the initial amounts. Ward and Bej[16] reported that co-existence of >2,500-fold nonspecific DNA caused a coprecipitation effect and inhibited the PCR in the real-time PCR system for *V. parahaemolyticus* targeting *tlh*, ORF8, *tdh*, and *trh* genes. However, in this experiment, we did not use such a huge amount of DNA as the competitor. The occurrence of PCR competition by *V. rotiferianus* DNAs was the major cause of the underestimation because RTVHAF and RTVHAR primers were completely matched to the *V. rotiferianus* sequence. To increase the specificity and sensitivity of the real-time PCR, a more specific primer set showing high discrimination will be required in further study.

The effect of DNA preparation methods on the sensitivity of the real-time PCR was examined. The detection limits for the boiling method, chelating resin method and DNA Purification kit method were 1.2×10^3 CFU/ml, 1.2×10^2 CFU/ml and 1.2×10^5 CFU/ml, respectively. The detection limit for the chelating resin method was higher than the other methods. It was reported that since chelating resins had a high affinity to polyvalent metal ions which catalyze the degradation of DNA, the resins had the effect of reducing the PCR inhibitor[15]. In addition, chelating resin has effects on cell lysis and DNA dissociation due to the strong alkalinity of the solution. The boiling method was the simplest, but it did not remove PCR inhibitors. Conversely, as the DNA extraction kit had several steps for extraction and handling, losses of DNA in each extraction step were large due to the low detection limit (1.2×10^5 CFU/ml). Furthermore, the detection limit using chelating resin DNA preparation from the seeded autoclaved seawater in three independent experiments was $7.3\pm1.3\times10^1$ CFU/ml, showing a positive coefficient (R^2= 0.955) (Fig. 1.). A slope of −3.36 was obtained, the efficiency of the curve being 98.4%.

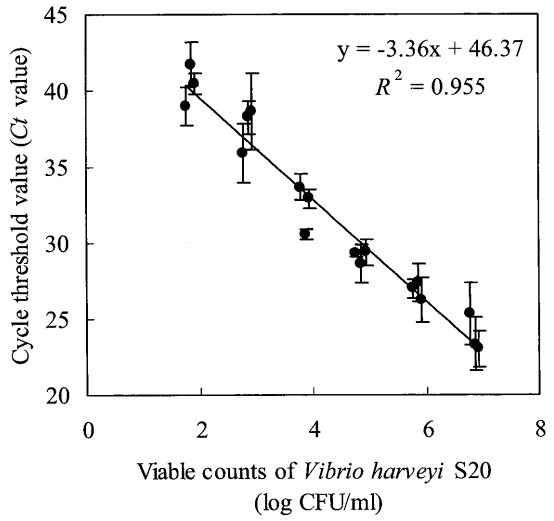

Fig. 1. Sensitivity of the real-time PCR using an exponentially grown *Vibrio harveyi* S20 extracted with the chelating resin method. The error bars indicate the standard deviation in triplicate for three independent experiments.

Under the nutrient limited buffered-ASW at 20°C, direct counts of *V. harveyi* were 1.6×10^8, 1.2×10^8, and 1.2×10^8 cells/ml after incubation of 0, 7, and 14 days, respectively. On the other hand, viable bacterial counts were decreased one log from 9.1×10^7 to 2.2×10^6 CFU/ml during 14 days incubation. The detection limits (10^2 cells/ml) of direct counts were not changed during the 14 day experiment, showing determination coefficients (R^2) of 0.971, 0,976, and 0.747, at 0 day, 7 days, and 14 days, respectively. It was reported that nonculturable cells of *V. vulnificus* after incubation in ASW at 4°C were detected by real-time PCR, unless complete degradation of membranes or loss of nucleic acid occurred[3]. However, after 14 days, the determination coefficient (R^2) was low at 0.747, indicating that the efficiency of extraction had declined. This result supported that such starved cells in the environment are unlikely to be lysed[3], causing underestimation of the real-time quantification.

Seawater samples collected at an abalone farm in 2005 were treated by chelating resin, and subsamples were used for quantitative real-time PCR. A *V. harveyi*-specific amplification was observed from breeding seawater collected on 23 August 2005, when a mass mortality of abalone occurred (Table 3). The *Ct* values were obtained with two (37.29 and 37.53) out of three samples examined. The *V. harveyi* counts were presumed to be less than 1.7×10^2 CFU/ml. The abundance determined by real-time PCR showed the same value as that determined by the colony PCR detection (2.8×10^2 CFU/ml). However, two samples determined as 10^1 CFU/ml by the colony PCR method could not be detected by the real-time PCR method (Table 3), perhaps because of PCR competition from other related bacteria as shown in Table 2.

We developed a *V. harveyi*-specific quantitative real-time PCR method targeting the 16S rRNA gene. This method could be applied to *V. harveyi* populations in an aquatic environment within five hours. The specificity of *V. harveyi* was obtained at 68°C of annealing and extension. An increase of the annealing temperature was predicted to reduce the detection limits of the quantitation of *V. harveyi*. Using the chelating resin method, the amount of water sample was 1 ml and the detection limit reached 6.0×10^1 CFU/ml with high sensitivity. In fact, this method was able to quantify more than 10^2 CFU/ml of *V. harveyi* in a sample of rearing seawater when abalone mortality occurred. Detection of *V. harveyi* from 1 ml of water sample allows i) simple and rapid sample preparation, and ii) investigation of the microecology of *V. harveyi* in many environmental samples. Therefore, the method is suitable for on-site quantitative PCR of *V. harveyi*. Establishment of a quantitative *V. harveyi*-specific detection method would be useful for examining the ecology of *V. harveyi* in the natural environment. In future, a further increase of the detection limit will be necessary to examine the proliferation of *V. harveyi* before outbreaks. For early warning monitoring of outbreaks, developments such as more specific oligonucleotide and sample preparation that allow detection of low numbers of *V. harveyi* will be required.

Table 3. Detection of *Vibrio harveyi* from seawater samples collected from an abalone farm

Date	Sample	Estimated viable counts by real-time PCR[a] (CFU/ml)	Viable plate counts (CFU/ml)
Aug. 9, 05	Coastal seawater	ND[b]	ND
	Supplying seawater	ND	0.005
	Breeding seawater	ND	ND
Aug. 23, 05	Coastal seawater	ND	65
	Supplying seawater	ND	11
	Breeding seawater	<172	280
Sep. 9, 05	Coastal seawater	ND	44
	Supplying seawater	ND	5.1
	Breeding seawater	ND	62

[a] Estimated viable counts by standard curve. y=–3.23x+44.82; x=log (CFU counts), y=(Practical *Ct*)
[b] ND, not detected.

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