Microbes Environ. Vol. 22, No. 1, 1–10, 2007
http://wwwsoc.nii.ac.jp/jsme2/

Microbes and Environments

Improved One-step Colony PCR Detection of *Vibrio harveyi*

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(Received September 13, 2006—Accepted October 20, 2006)

Vibrio harveyi strains are pathogenic to a wide range of marine fish and shellfish, having a significant negative economic impact on aquaculture worldwide. However, a reliable and rapid method of detecting *V. harveyi* has yet to be established. We aimed to construct an improved one-step detection method for *V. harveyi*. Reanalysis of 16S rRNA gene sequences of type strains of *V. harveyi* revealed a unique consensus region compared to related *Vibrio* species. Using a VHARF-VHARR primer set from this region, a *V. harveyi*-specific PCR-based detection method was established and could differentiate *V. harveyi* from related species such as *V. campbellii*, *V. rotiferianus*, and *V. alginolyticus*. Furthermore, the new method (optimal amplification with 20 core cycles of (94°C–30 s, 60°C–30 s, 72°C–30 s)) could be applied to the identification of *V. harveyi* strains of colonies. This PCR was able to detect *V. harveyi* grown on plates in the environment within 3 days without bacterial isolation, DNA extraction, or the assistance of biochemical tests. The specificity and rapidity of the detection is reliable enough to understand the ecology of *V. harveyi* in environments, and to predict outbreaks of mass mortality caused by *V. harveyi* in aquaculture.

Key words: *Vibrio harveyi*, 16S rRNA gene, colony PCR, detection

Vibrio harveyi strains have been reported as causing mortalities in shark[14,15], grouper[49], flounder[40], salmonids[50], abalone[28,30] and pearl oyster[34]. Therefore, they are considered to be pathogens of various fish and shellfish[4,5]. In addition, shrimp farms have sustained serious economic losses caused by mass mortalities due to *V. harveyi* or luminescent vibrio[3,7,21–24,36,37,41]. Moreover, an outbreak of mass mortality was reported in one of the biggest abalone farms in Japan, the occurrence of which was attributed to *V. harveyi* infections (Sawabe *et al.*, manuscript in preparation).

The ecology and epidemiology of *V. harveyi* in aquacultural facilities, including abalone farms, is not fully understood. Elucidation of the bacterial ecology may enable us to predict future outbreaks of mass mortality and to establish strategies for minimizing the death caused by these outbreaks. As the mortality has occurred suddenly in aquaculture, an extremely rapid and simple species-specific identification system is needed to diagnose the *V. harveyi* infection.

Recently, various housekeeping genes and virulence-associated genes have been used as genetic markers of identification and phylogeny of bacteria[29]. However, such genes are not available for all bacterial species even in the family *Vibrionaceae*, except 16S rRNA gene sequences. Eighty species belonging to *Vibrionaceae* can basically be classified based on 16S rRNA gene sequences[33,45,46], the only gene sequences available for every species of *Vibrionaceae* in public databases. Therefore, the 16S rRNA gene is still the best gene for minimizing false-positive reactions in the development of species-specific PCR detection. *V. harveyi* is known to be very similar to many related species, thus it has been difficult to detect it at the species level by targeting the 16S rRNA gene. Oakey *et al.*[32] reported PCR identification of *V. harveyi* based on the 16S rRNA gene. However, this method of detection requires the phenotypic characterization of swarming on blood agar and acetoin production of PCR-positive strains due to the weak specificity of the PCR primer set for differentiating *V. harveyi* from *V. alginolyticus*. The extra-steps of bacterial isolation and two bio-

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chemical tests are time-consuming and laborious, which diminishes the rapidity of the detection.

Recently, Gauger and Gómez-Chiarri[11] reported that the sequence of a *V. harveyi* type strain (X74706) in the database had errors. In the present study, 16S rRNA gene sequences of *V. harveyi* type strains were reanalyzed and the region of errors reported by Gauger and Gómez-Chiarri[11] was confirmed. This region was likely to be useful for species-specific detection of *V. harveyi* strains. The purpose of this work is to develop an improved one-step colony PCR-based method of detecting *V. harveyi* using this region as a primer sequence.

Materials and Methods

Bacterial strains and DNA extraction

V. harveyi strains NCIMB1280^T and LMG4044^T, and the abalone pathogenic *V. harveyi* strains S20 and S35 (isolated from dead abalone) were subjected to 16S rRNA gene sequencing. A total of 32 species, including seven strains of *V. harveyi* (Table 1), were used to evaluate the specificity of the PCR. All strains were cultured on ZoBell 2216E agar and genomic DNA of each strain was extracted using a Wizard Genomic DNA Purification kit (Promega, Madison, USA).

16S rRNA gene sequence determination

Nearly full-length 16S rRNA gene sequences for *V. harveyi* strains NCIMB1280^T, LMG4044^T, S20, and S35 were determined using six sequence primers (24F, 530F, 1100F, 520R, 920R, and 1509R) according to Sawabe *et al.*[39]. The sequences of these four *V. harveyi* strains were deposited in the DDBJ/EMBL/GenBank database under AY750575 to AY750578, respectively.

V. harveyi species-specific PCR primer design

16S rRNA gene sequences from *V. harveyi* strains including re-determined sequences in this study, and closely related species deposited in GenBank (Table 2), were aligned with the ClustalX program[47]. We found possible conserved regions in *V. harveyi* strains, and designed a candidate primer set consisting of VHARF (5'-CCGCATAATACCTACGGGTC-3') and VHARR (5'-ACCCGAAGTGCTGGCAAACA-3'), which was expected to generate a 967 bp fragment. The specificity of this primer set was predicted by using the Check Probe program of Ribosomal Database Project II (http://rdp8.cme.msu.edu/html/)[25].

Conditions for PCR

Strains listed in Table 1 were used for PCR with the VHARF-VHARR primer set. Genomic DNA (25 ng) was added to reaction mixtures (total 25 μl) containing 0.2 μM of each primer, 1×PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, and 0.625 U of *Taq* polymerase (Promega, Madison, USA). GoTaq Green master mix (Promega, Madison, USA), in which a loading dye was pre-mixed, as a PCR mixture (total 25 μl) containing 0.2 μM of each primer was also used. The reaction was performed using a 9700 type thermal cycler (Applied Biosystems, Foster City, USA). General PCR consisted of initial denaturation at 94°C for 3 min followed by 15, 20, or 25 core cycles of (94°C–30 s, 60 or 63°C–30 s, and 72°C–30 s). The general touch-down conditions consisted of short stepwise cycles with a decrease in the annealing temperature. A (5-3-3-3-25) touch-down cycle consisted of a hot start for 5 min at 94°C followed by 5 cycles of 40 s at 94°C, 30 s at 67°C, and 20 s at 72°C, 3 cycles with the same physical conditions except that the annealing temperature was 64°C, 3 cycles with an annealing temperature of 61°C, 3 cycles with an annealing temperature of 59°C, and 25 cycles with an annealing temperature of 55°C[27]. A (3-3-14) consisted of a hot start for 3 min at 94°C followed by 3 cycles of 40 s at 94°C, 30 s at 65°C, and 20 s at 72°C, 3 cycles with the same conditions except that the annealing temperature was 62.5°C, and 14 cycles with an annealing temperature of 60°C. A (3-3-9) and a (5-5-5) were performed according to modifications of a (3-3-14). The PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels. Conditions for *V. harveyi*-specific PCR were optimized based on obtaining single clear amplicons in the shortest possible reaction time. These are important factors in the application of the PCR to many environmental isolates in the future. Specificity tests were performed in duplicate.

Construction of colony PCR

The *V. harveyi* strains NCIMB1280^T, LMG4044^T, S20, S35, and LMG7890 (previously known as *V. carchariae*), *V. nigripulchritudo* LMG3896^T, and *V. cincinnatiensis* LMG7891^T were grown on ZoBell2216E agar plates for 2 days at room temperature. Part of the colony formed on the medium was used as a template. The colony PCR was examined using both direct and indirect reactions. In the direct reaction, a small amount of colony was picked using autoclaved 10-μl tips, and added directly to the PCR mixture. In the indirect reaction, a small amount of colony was first suspended in 10 μl of autoclaved distilled water and

Table 1. Specificity of the *Vibrio harveyi* species-specific PCR amplification in the selected optimum PCR

Bacterial istrains	General PCR (20 cycles)		Touch-down PCR
	60°C annealing temp[a]	63°C annealing temp[a]	(3-3-14)[a]
V. harveyi S20	+	+	+
V. harveyi S35	+	+	+
V. harveyi LMG4044^T	+	+	+
V. harveyi NCIMB1280^T	+	+	+
V. harveyi LMG7890[b]	+	+	+
V. harveyi LMG19643[c]	+	+	+
V. harveyi LMG19714[c]	+	+	+
Phb. damasela subsp. *damasela* LMG7892^T	−	−	−
V. nigripulchritudo LMG3896^T	−	−	−
V. cincinnatiensis LMG7891^T	−	−	−
G. hollisae LMG17719^T	−	−	−
Phb. leiognathi LMG4228^T	−	−	−
V. gazogenes ATCC29988^T	−	−	−
V. scophthalmi LMG19158^T	−	−	−
V. splendidus LMG19031^T	−	−	−
V. campbellii LMG11216^T	−	−	−
V. rotiferianus LMG21460^T	−	−	−
V. proteolyticus LMG3772^T	−	−	−
V. anguillarum LMG4437^T	−	−	−
V. ordallii LMG13544^T	−	−	−
V. aestuarianus LMG7909^T	−	−	−
V. parahaemolyticus LMG2850^T	−	−	−
V. alginolyticus LMG4409^T	−	−	−
V. natriegens LMG10935^T	−	−	−
V. pelagius ATCC25916^T	−	−	−
V. navarrensis LMG15976^T	−	−	−
V. mediterranei LMG19703	−	−	−
V. tapetis LMG19706^T	−	−	−
V. mediterranei LMG11258^T	−	−	−
V. orientalis LMG7897^T	−	−	−
V. tubiashii LMG10936^T	−	−	−
V. nereis LMG3895^T	−	−	−
V. diazotrophicus LMG7893^T	−	−	−
V. furnissii LMG7910^T	−	−	−
V. fluvialis LMG7894^T	−	−	−
V. vulnificus LMG13545^T	−	−	−
V. metschnikovii LMG11664^T	−	−	−
V. mytili LMG19157^T	−	−	−
V. halioticoli IAM14596^T	−	−	−

[a] Results were obtained in two independent experiments.
[b] *V. harveyi* LMG7890 had been classified as *V. carchariae* previously[14].
[c] *V. harveyi* LMG19643 and LMG19714 have been classified as *V. trachuri* previously[18].

Table 2. Alignment of a part of the 16S rRNA gene sequence of *Vibrio harveyi* and related species

Species	Strains[a]	Sequences[b]									
		1134									1143
		5'									3'
V. harveyi	NCIMB1280^T (AY750575[1]), LMG4044^T (AY750576[1]), ATCC14126^T (AF426811[3], AF426825[3]), S20 (AY750577[1]), S35 (AY750578[1]), ATCC35084 (AF426814[3], X74693[3]), LMG19643 (AJ312382[7]), flounder (AF134581[5]), USA (AF426805[3]), Taiwan (AF426806[3]), VIB 660 (AF426808[3]), VIB 645 (AF426812[3]), VIB 651 (AF426815[3]), VIB 573 (AF426817[3]), VIB 654 (AF426819[3]), VIB 570 (AF426822[3]), VIB 411 (AF426823[3])	A	C	T	T	C	G	G	G	T	—
Vibrio spp.[c]	X56578[2] and X74706[4] from ATCC14126^T	G	A	G	·	A	A	T	·	·	C
	VIB 655 (AF426807[3]), VIB 657 (AF426809[3]), VIB 656 (AF426810[3]), VIB 350 (AF426813[3]), VIB 400 (AF426816[3]), VIB 697 (AF426818[3]), VIB 403 (AF426820[3]), VIB 394 (AF426821[3])										
V. rotiferianus	LMG21460^T (AJ316187[6])	G	A	G	·	A	A	T	·	·	C
V. campbellii	ATCC25920^T (AF46824[3], X56575[2], X74692[4])	G	A	G	·	A	A	T	·	·	C
V. alginolyticus	ATCC17749^T (X56576[2], X74690[4])	G	A	G	·	A	A	T	·	·	C
V. parahaemolyticus	ATCC17802^T (X56580[2], X74720[4])	G	A	G	·	A	A	T	·	·	C
V. natriegens	ATCC14048^T (X74714[4])	G	A	G	·	A	A	T	·	·	C
V. proteolyticus	ATCC15338^T (X56579[2])	N	·	G	·	A	A	T	·	G	C
	ATCC15338^T (X74723[4])	·	·	G	·	A	A	T	·	G	T
Escherichia coli	CFT073 (AE014075[8])	G	—	G	·	·	C	·	·	C	C

[a] 1. This study, 2. Dorsch *et al.*[10], 3. Gauger and Gomez-Chiarri[11], 4. Ruimy *et al.*[38], 5. Soffientino *et al.*[40], 6, 7. Thompson *et al.*[43,44], 8. Welch *et al.*[48].

[b] Part of the sequence corresponding to positions 1134 to 1143 of *Escherichia coli* is shown. Nucleotides identical with those of *V. harveyi* type strain AY750575 are represented by dots.

[c] Most strains have been identified as *V. harveyi*.

vortexed for 20 seconds, and then 1 μl of the suspension was added to the PCR mixture. This experiment was repeated five times and the optimum PCR conditions were determined based on the highest detection rate.

Results

Comparison of 16S rRNA gene sequences

Forty 16S rRNA gene sequences including *V. harveyi* strains and phylogenetically related *Vibrio* spp. retrieved from databases were aligned and compared (Table 2). Major nucleotide variations were observed in the region corresponding to positions 1134–1143 of *Escherichia coli* (AE014075). Sequences of the region (5'-ACTTCGGGT-3') in both strains of *V. harveyi* NCIMB1280^T and LMG4044^T (AY750575 and AY750576), *V. harveyi* ATCC14126^T (AF426811 and AF426825), *V. harveyi* S20 and S35, *V. harveyi* ATCC35084 (previously named as *V. carchariae*), *V. harveyi* LMG19643 (previously named as *V. trachuri*), and the other environmental isolates of *V. harveyi*[11,40] were identical (Table 2). In contrast, distinct differences in the region (5'-GAGTAATGTC-3') in X56578 and X74706 from *V. harveyi* ATCC14126^T, *V. rotiferianus* LMG 21460^T, *V. campbellii* ATCC 25920^T, *V. alginolyticus* ATCC17749^T, *V. parahaemolyticus* ATCC17802^T, *V. natriegens* ATCC14048^T, *V. proteolyticus* ATCC15338^T, and environmental isolates of *V. harveyi*[11] were observed.

Primer design

Since the sequence corresponding to *E. coli* positions 1134–1143 belonged to a highly preserved region in *V. harveyi* strains, this region was used as a part of the VHARR sequence. VHARF was selected for *E. coli* positions 175–194 due to both its specificity for *V. harveyi* strains and the number of mismatches against non-*V. harveyi* strains (Table 3). The specificity of both primers was evaluated with the RDP-II Check Probe program[25] (Table 3). The VHARF sequence had more than two nucleotide mismatches against

Table 3. Comparison of VHARF-VHARR primer sequences against *Vibrio harveyi* and *Vibrio* spp.

Species	Strains	NM[a]	VHARF primer[b]	NM[a]	VHARR primer[b]
			175 5' C C G C A T A A T A C C T A C G G G T C 3' 194		1142 5' A C C C G A A G T G C T G G C A A A C A 3' 1123
V. harveyi	S20 (AY750577), S35 (AY750578), ATCC35084 (X74693), LMG19643 (AJ312382)	0	G G C G T A T T A T G G A T G C C C A G	0	T G G G C T T C A C G A C C G T T T G T
	NCIMB1280^T (AY750575), LMG4044^T (AY750576)	1	· · · · · · · · · · · · · · · W · · · · · ·	0	· ·
V. nigripulchritudo	CIP103195^T (X74717)	3	· · · · · · · · · · · C · · A · · · G · ·	0	· ·
V. cincinnatiensis	ATCC35912^T (X74698)	4	· · · · · · C · · · C · · A · · · G · ·	0	· ·
G. hollisae	ATCC33564^T (X56583)	4	· · · · · · · · · · · C · · A · · · G · C	0	· ·
G. hollisae	ATCC33564^T (X74707)	5	· · · · · · · · · · · C A · · A · · · T G ·	0	· ·
V. proteolyticus	ATCC15338^T (X56579)	2	· · · · · · · · · · · C · · · · · · · · G ·	6	G · T A A · G · N · · · · · · · · · ·
V. proteolyticus	ATCC15338^T (X74723)	3	· · · · · · · · · · · C · · A · · · G · ·	5	G · T A A · G · · · · · · · · · · · ·
V. rotiferianus	LMG21460^T (AJ316187)	2	· · · · · · · · · · · C · · · · · · · · G ·	6	· · T A A · G A G · · · · · · · · · ·
V. campbellii	ATCC25920^T (X74692)	2	· · · · · · · · · · · C · · · · · · · · G ·	6	· · T A A · G A G · · · · · · · · · ·
V. campbellii	ATCC25920^T (X56575)	3	· · · · · · · · · · · C - · · · · · · · G ·	6	· · T A A · G A G · · · · · · · · · ·
V. parahaemolyticus	ATCC17802^T (X74720)	3	· · · · · · C · · C · · · · · · · · G ·	6	· · T A A · G A G · · · · · · · · · ·
V. parahaemolyticus	ATCC17802^T (X56580)	5	· · · · · · C · · · C · · A · · · G · A	6	· · T A A · G A G · · · · · · · · · ·
V. alginolyticus	ATCC17749^T (X74690)	3	· · · · · · C · · C · · · · · · · · G ·	6	· · T A A · G A G · · · · · · · · · ·
V. alginolyticus	ATCC17749^T (X56576)	4	· · · · · · · · · - C - · · · · · · · G ·	6	· · T A A · G A G · · · · · · · · · ·
V. natriegens	ATCC14048^T (X74714)	3	· · · · · · C · · C · · · · · · · · G ·	6	· · T A A · G A G · · · · · · · · · ·
Escherichia coli	CFT073 (AE014075)	9	· · · · · · · · · G C A · C G T T · T G ·	8	C · · C · · G - G · · · · · · · · · G T ·

[a] Numbers of mismatches (NM) are given by comparison between the sequence of the target region and each primer.
[b] Nucleotides identical with those of *V. harveyi* S20 strain are represented by dots.

non-*V. harveyi* strains. Conversely, the VHARR sequence completely matched the sequences of *V. nigripulchritudo*, *V. cincinatiensis*, and *Glimontia hollisae*, but more than five mismatches were found against sequences of *V. harveyi*-related species (*V. campbellii*, *V. rotiferianus*, *V. alginolyticus*, and so on) (Table 3). Based on the results of this estimation, it was suggested that the annealing temperature should be increased to enhance the specificity of the primers used in the detection of *V. harveyi*.

Determination of optimum PCR conditions

The specificity of the VHARF-VHARR primer set was first tested against four strains, *V. harveyi* LMG4044^T, *V. harveyi* S20, *V. nigripulchritudo* LMG3896^T, and *V. cincinnatiensis* LMG7891^T. In a general PCR consisting of 25 cycles including an annealing temperature at 60°C, the amplified PCR products were detected in both *V. harveyi* and *V. nigripluchritudo*. In contrast, these products were only obtained from *V. harveyi*-related strains, in 25 cycles when the annealing temperature was set at 63°C. By reduc-

Table 4. Accuracy of the colony PCR method for identifying *Vibrio harveyi*

Species	General PCR 60°C annealing temp		General PCR 63°C annealing temp		Touch down PCR (3-3-14)	
	Direct detection rate (%)[a]	Indirect detection rate (%)	Direct detection rate (%)	Indirect detection rate (%)	Direct detection rate (%)	Indirect detection rate (%)
V. harveyi[b]	9/25 (36)	25/25 (100)	3/25 (12)	24/25 (96)	9/25 (36)	24/25 (96)
V. nigripulchritudo LMG3896^T	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
V. cincinnatiensis LMG7891^T	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)

[a] Detection rate was determined as the ratio of the number of positive colonies to total number of colonies tested.
[b] *V. harveyi* S20, *V. harveyi* S35, *V. harveyi* LMG4044^T, *V. harveyi* NCIMB1280^T, and *V. harveyi* LMG7890 were used for the colony PCR.

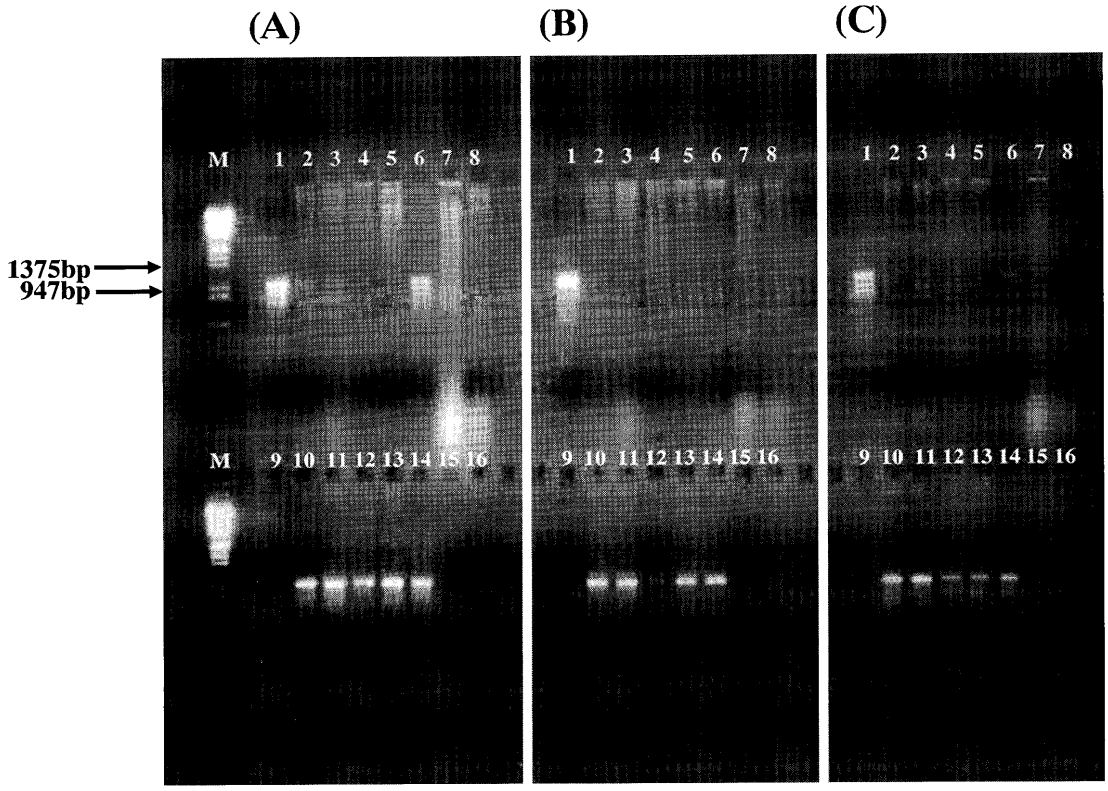

Fig. 1. Electrophoresis of colony PCR (A); General PCR with initial denaturation at 94°C for 3 min and 20 cycles of (94°C–30 s, 60°C–30 s, 72°C–30 s), (B); General PCR with initial denaturation at 94°C for 3 min and 20 cycles of (94°C–30 s, 63°C–30 s, 72°C–30 s), (C); Touch-down PCR (3-3-14). Lanes: 1, *V. harveyi* S20 (Genome DNA); 2, *V. harveyi* S20 (direct); 3, *V. harveyi* S35 (direct); 4, *V. harveyi* LMG4044^T (direct); 5, *V. harveyi* NCIMB1280^T (direct); 6, *V. harveyi* LMG7890 (direct); 7, *V. nigripulchritudo* LMG3896^T (direct); 8, *V. cincinnatiensis* LMG7891^T (direct); 9, negative control (no genomic DNA); 10, *V. harveyi* S20 (indirect); 11, *V. harveyi* S35 (indirect); 12, *V. harveyi* LMG4044^T (indirect); 13, *V. harveyi* NCIMB1280^T (indirect); 14, *V. harveyi* LMG7890 (indirect); 15, *V. nigripulchritudo* LMG3896^T (indirect); 16, *V. cincinnatiensis* LMG7891^T (indirect); M: size marker (lambda DNA/*Eco*RI+*Hin*dIII marker).

ing the number of cycles to 20 or less, the total reaction time could be reduced to less than one hour. With the shorter PCR, a specific band of *V. harveyi* was observed at annealing temperatures of both 60 and 63°C, which was much clearer in the PCR with 20 cycles than 15 cycles. Consequently, 20 core cycles of (94°C–30 s, 60 or 63°C–30 s, 72°C–30 s) were selected as optimum conditions for the *V. harveyi*-specific PCR.

In a touch-down PCR (5-3-3-3-25), bands were obtained from *V. harveyi*, *V. nigripluchritudo*, and *V. cincinnatiensis*. The specificity for *V. harveyi* was increased with the other three reaction profiles (3-3-14), (3-3-9), and (5-5-5). Since the intensity of the band against *V. harveyi* was strongest with (3-3-14), the (3-3-14) profile was determined to be the optimum for touch-down PCR.

The PCR under optimum conditions was able to identify seven strains of *V. harveyi* from 32 species in two independent experiments (Table 1). This means that the primer specificity was high enough to reduce the number of false positive reactions.

Colony PCR

A sufficient number of bacterial cells for the colony PCR was obtained after 2 days incubation at room temperature. The specificity and reproducibility for *V. harveyi* of direct and indirect reactions were compared among the three PCR cycles with the above conditions (Table 4, Fig. 1). In the direct reactions, amplification rates for *V. harveyi* strains were low, 12–36%. Conversely, the amplification rate of *V. harveyi* strains was 100% in the indirect reactions with the PCR consisting of 20 core cycles of (94°C–30 s, 60°C–30 s, 72°C–30 s). The amplification rates for *V. harveyi* strains were 96% with the other two PCR conditions. Therefore, the use of 20 core cycles of (94°C–30 s, 60°C–30 s, 72°C–30 s) was found to be the most specific and reproducible way to identify *V. harveyi* strains with the colony PCR method.

Discussion

Different sequences in a region corresponding to *E. coli* positions 1134–1143 between *V. harveyi*-type strains (AY750575 and AY750576) and sequences of *V. harveyi*-type strains (X56578 and X74706) were confirmed (Table 2). This region was reported to contain sequence errors by Gauger and Gómez-Chiarri[11]. Therefore, the region corresponding to *E. coli* positions 1134–1143 was used as a part of the VHARR sequence to differentiate *V. harveyi* from related species such as *V. campbellii*, *V. rotiferianus*, and *V. alginolyticus*. The VHARF region is rather specific to *V. harveyi* having more than 2 nucleotide mismatches with other *Vibrio* species. Using a highly conserved region of *V. harveyi* strains, we succeeded in constructing a PCR-based detection system that was highly specific for *V. harveyi* strains (Table 1). The 16S rRNA gene copy number in *V. harveyi* has never been reported. However, those of *V. cholerae*[16], *V. natriegens* (Ribosomal RNA Operon Copy Number Database; http://rrndb.cme.msu.edu/rrndb/servlet/controller), *V. parahaemolyticus*[26], *V. vulnificus*[6], and *V. fischeri*[31] range from 8 to 13. As *V. harveyi* is a fast growing bacterium and related to *V. parahaemolyticus*, we assumed that *V. harveyi* might have the same copy numbers in *Vibrionaceae*. Therefore, differences in copy numbers among the *Vibrionaceae* species examined in this study may not affect the specificity of this *V. harveyi*-specific PCR.

In addition, it was possible to apply the general PCR profile determined in this study to a colony PCR method that used the bacterial colony as a template. To understand the distribution of *V. harveyi* in the environment, huge amounts of isolates must be identified. In bacterial ecology, it is always necessary to establish simple and rapid techniques with accurate species specificity without the need for isolation[42]. Colony PCR would be a powerful tool as it is a simple and rapid method and does not require the isolation and extraction of nucleic acid from each strain. Using thiosulfate citrate bile saccharose (TCBS) agar (Nissui pharmaceutical, Tokyo, Japan: 5 g yeast extract, 10 g peptone, 17 g sucrose, 10 g sodium thiosulfate, 10 g sodium citrate, 3 g sodium cholate, 1 g iron (II) citrate, 10 g sodium chloride, 5 g bile, 0.04 g bromothymol blue, 0.04 g thymol blue and 15 g agar per liter of distilled water) medium for the first plate culture and the colony PCR, the total amount of time needed to detect *V. harveyi* strains in environmental samples including plate cultures was less than 3 days. In contrast, the PCR-based detection method developed by Oakey *et al.*[32] needed at least 10 days including one week for isolating the strain before the PCR, and a few days for phenotypic characterization (swarming an VP reaction) after the PCR. As *V. harveyi* is pathogenic to a wide range of marine fish and shellfish[4,5], the mechanism of mass mortality must be analyzed in future studies. Therefore, one advantage of the specific colony PCR is to obtain a great number of *V. harveyi* strains from the environment. These *V. harveyi* strains may also be of use in research on pathogenesis, population biology, and evolution.

In the present study, an indirect reaction using the general PCR cycle showed the best specificity and reproducibility (Table 4, Fig. 1). In general, the fastest methods involved direct reactions in which the culture was added directly to the PCR mixture. However, because the amplification rates of *V. harveyi* were low in the direct reactions (Table 4), they could not be applied to the colony PCR. The low amplification rates could be attributed to an excessive amount of DNA or PCR inhibiting materials that were carried into the tube. It is reported that proteins and complex carbohydrates

in bacterial colonies absorb DNA[2] and DNA polymerase[20], respectively, and inhibit the PCR. Conversely, amplification rates of *V. harveyi* were high in indirect reactions (Table 4). These results indicate that the indirect reaction might be used to dilute possible PCR inhibitors and disrupt *V. harveyi* cells.

Several PCR methods[8,9,17,19] to detect *V. harveyi* other than those of Oakey *et al.*[32] have been reported. Iwamoto *et al.*[19], and Hernández and Olmos[17] reported the specific detection of *V. harveyi* using genes that targeted *Pst*I-*Hin*dIII fragments in genomic DNA and *luxN*, respectively. However, these methods did not have enough specificity to evaluate with a few *V. harveyi* strains and other bacterial strains. Furthermore, PCR analysis based on the *toxR* gene[8] was not specific enough to detect all *V. harveyi* strains. PCR detection based on the hemolysin (*vhh*) gene was specific for most of the *V. harveyi* strains tested, however, non-specific amplification was also observed in a *V. harveyi* environmental isolate[9].

Discrimination of environmental isolates belonging to *V. harveyi*, *V. campbellii*, and *V. alginolyticus* is even more difficult because these species share highly similar phenotypic traits and have almost identical 16S rRNA gene sequences[1,11–13]. In this paper, the sequences of VIB 655, VIB 657, VIB 656, VIB 350, VIB 400, VIB 697, VIB 403, and VIB 394 had a different region from *V. harveyi*-type sequences corresponding to positions 1134–1143 of *E. coli* (Table 2). While VIB 655, VIB 657, VIB 656, VIB 697, VIB 403, and VIB 394 were reported to be more closely related to *V. campbellii*[11], VIB 350 and VIB 400 were more similar to *V. mediterranei* and *V. rumoiensis*, respectively[11]. The newly established improved PCR-based method of identifying *V. harveyi* in this study could distinguish *V. harveyi* from phenotypically closely related species such as *V. campbellii*, *V. mediterranei*, and *V. rumoiensis*. Furthermore, *V. harveyi* strains detected in environmental samples collected from abalone farms using the colony PCR were identified as *V. harveyi* based on other housekeeping genes phylogeny (data not shown). This proved that this method would decrease the number of false-positive reactions.

Since *V. harveyi* ATCC35084 and *V. harveyi* LMG19643 were formerly classified as a *V. carchariae*-type strain and *V. trachuri*-type strain, respectively, they were considered to be species that are different from *V. harveyi*[14,18]. Later, *V. carchariae* was reclassified as a junior synonym of *V. harveyi* by comparison with AFLP, DNA-DNA hybridization, and ribotyping[35]. *V. trachuri* was also reclassified as a junior synonym based on polyphasic taxonomy[44]. In our analysis, we confirmed that 16S rRNA gene sequences of *V. harveyi*-type strains (AY750575, AY750576, AF426811, and AF426825), *V. harveyi* ATCC35084 (AF426814 and X74693), and *V. harveyi* LMG19643 (AJ312382) were identical. Furthermore, strong amplification signals were observed in *V. carchariae* and *V. trachuri* by the improved PCR method (Table 1). These results support that *V. harveyi*, *V. carchariae*, and *V. trachuri* are the same species. Accordingly, accurate 16S rRNA gene sequences for *V. harveyi* (AY750575, AY750576, AF426811, and AF426825) should be used for phylogenetic analysis in future studies. On the contrary, Alsina and Blanch[1] reported that *V. harveyi* and *V. carchariae* differed in the utilization of L-arabinose and melibiose, and xanthine decombination. Sawabe *et al.* (manuscript in preparation) also observed that *V. harveyi* strains pathogenic to abalone (S20 and S35) showed heterogeneous phenotypic traits in bioluminescence, urea degradation, and swarming mortality from *V. harveyi* LMG4044^T and LMG7890. However, these five strains of *V. harveyi* showing different phenotypes produced a single specific amplicon with the colony PCR method (Table 4, Fig. 1.). This suggested that the colony PCR established in this study could detect a broad range of phenotypically heterogeneous strains in *V. harveyi*.

In conclusion, sequence errors in the 16S rRNA gene of *V. harveyi* type strains (X56578 and X74706) were confirmed and this region was used to differentiate *V. harveyi* from related species. Using the VHARF-VHARR primer set, the newly established improved one-step colony PCR of *V. harveyi* was reliable in terms of distinguishing and identifying *V. harveyi* from closely related species without DNA extraction and any support from phenotypic tests. Using the new detection system, it should be possible in the future to rapidly identify *V. harveyi* strains among environmental isolates and to understand the ecology of *V. harveyi*, including pathogenic strains in detail. Based on the *V. harveyi* species-specific primer, a multiplex PCR system to select pathogenic strains will be needed. Moreover, combining the established colony PCR with a culture-independent technique to quantitate *V. harveyi* cells is an ideal methodology to evaluate *V. harveyi* abundance in the environment.

Acknowledgements

This work was supported by scientific grants from The Hokusui Society Foundation, Sapporo and The Institute of Fermentation, Osaka. We would also like to thank to Drs. J. Swings, D. Janssens, and C. Vereecke (Laboratory of Microbiology, University of Ghent and LMG culture collection) for providing *Vibrio* strains.

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