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16S rRNA targeted RT-PCR for the detection of *Vibrio penaeicida*, the pathogen of cultured kuruma prawn *Penaeus japonicus*

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ABSTRACT: *Vibrio penaeicida* is the causative bacterium of vibriosis in cultured kuruma prawn *Penaeus japonicus* in Japan. To develop a specific and sensitive method for the detection of the pathogen, a species-specific sequence in the 16S rRNA of *V. penaeicida* was determined and a polymerase chain reaction (PCR)-based method was devised on the basis of the sequence. Prior to sequencing, a part of the variable regions of the 16S rRNA was amplified by using primers designed from 2 conserved regions according to previously reported data on Vibrionaceae. The region of the 16S rRNA (nucleotide numbers 440 to 490 in *Escherichia coli* 16S rRNA) obtained by this procedure was found to be species-specific for *V. penaeicida*. It was confirmed that PCR and RT (reverse transcription)-PCR amplifications with a sense primer designed from the *V. penaeicida*-specific sequence were both able to differentiate *V. penaeicida* from other prawn-pathogenic vibrios. 16S rRNA-targeted RT-PCR was demonstrated to have 100 times higher sensitivity than 16S rDNA-targeted PCR and 10 fg of total nucleic acids extracted from cultured bacterial cells was sufficient to yield the visible fragment in gel electrophoresis. These results indicate that RT-PCR amplification with this primer is useful for specific and sensitive detection of *V. penaeicida*.

KEY WORDS: *Vibrio penaeicida* · rRNA · PCR · Kuruma prawn · Vibriosis

INTRODUCTION

It is well known that several species of the genus *Vibrio*, for example *Vibrio parahaemolyticus* and *V. alginolyticus*, cause septicemia in marine crustaceans resulting in economic damage, particularly among cultured penaeid prawns (Brock & Lightner 1990, Stewart 1993). Vibriosis has also been the most serious disease problem in cultured kuruma prawn *Penaeus japonicus* in Japan, and one species of *Vibrio* (*Vibrio* sp. PJ) has been reported as the causative agent of the disease (Takahashi et al. 1985, de la Peña et al. 1993). This causative bacterium was confirmed to be a new species and named *Vibrio penaeicida* sp. nov. (Ishimaru et al. 1995). As is the case with other vibrios pathogenic to prawn and shrimp, *V. penaeicida* was demonstrated to be a part of the ubiquitous microflora in apparently healthy kuruma prawns, and in their culture environment, which will produce vibriosis in a compromised (stressed) host (de la Peña et al. 1992). Recently, the dynamics of *V. penaeicida* in kuruma prawns experimentally infected by oral intubation was studied by a viable cell count method and an indirect fluorescent antibody technique (de la Peña et al. 1995). The results of that study suggested that the pathogen probably multiplies in the stomach in the establishment stage of infection; however, exact site(s) for initial multiplication could not be determined due to abundant presence of other vibrios. To solve this problem, an alternative, more sensitive method to detect the pathogen is required.

Ribosomal RNA (rRNA) is present in high copy numbers in a growing bacterial cell and there are conserved and variable sequences in rRNA at the genus and species levels of bacteria (Woese 1987). These features make rRNA suitable as a target for hybridization using DNA probes complementary to rRNA and

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polymerase chain reaction (PCR) amplification. The 16S rRNA-targeted hybridization and PCR techniques have been applied for identification and detection of several fish pathogens (Rehnstam et al. 1989, Mattsson et al. 1993, Martínez-Picado et al. 1994, Toyama et al. 1994). In this study, a species-specific sequence in the 16S rRNA of *Vibrio penaeicida* was analyzed and PCR-based methods to detect 16S rDNA or rRNA of the pathogen were examined.

MATERIALS AND METHODS

Bacterial strains and culture condition. Seven strains of *Vibrio penaeicida* were isolated from diseased kuruma prawns in different places in Japan. Thirteen *Vibrio* species reported to be pathogenic to prawns, and *Escherichia coli*, were used for comparison, even though these strains were not isolated from prawns (Table 1). They were grown at 25°C for 18 to 20 h while being shaken in ZoBell's 2216E broth (0.5 g peptone, 0.1 g yeast extract, 0.01 g $FePO_4$, 100 ml filtered sea water) for *V. penaeicida* or in tryptic soy broth (Nissui) prepared with ½-diluted sea water for other vibrios and *Escherichia coli*.

Extraction of bacterial nucleic acids. Bacterial cultures were harvested by centrifugation at 10 000 × *g* for 10 min and the resultant pellets were lysed with 1% SDS/1 mg ml^{-1} proteinase K solution. Bacterial nucleic acids were extracted by a phenol-chloroform-isoamyl-alcohol (25:24:1, v/v/v) mixture and a chloroform-isoamylalcohol (24:1, v/v) mixture. The nucleic acids obtained were precipitated with sodium acetate (pH 5.2) and ethanol, and resuspended in water treated with diethyl pyrocarbonate (DEPC).

Sequencing of PCR products from 16S rDNA. An antisense primer, 5'-CCCGGGATCCGATTACCAGG-GTATCTAATC-3' (R-GEN), and a sense primer, 5'-CCGAATTCAGCAGTGTGGAATATTGCA-3' (F-GEN), were used for PCR amplification of a partial 16S rDNA. The R-GEN primer was complementary to a conserved region of 16S rRNA of Vibrionaceae (base positions 786 to 805 in *Escherichia coli* 16S rRNA; Kita-Tsukamoto et al. 1993) with an additional 10 base linker sequence (underlined) containing *Sma*I and *Bam*HI recognition sites. The F-GEN primer was complementary to another conserved region of 16S rRNA of Vibrionaceae (base positions 352 to 371) with a 7 base linker sequence (underlined) containing an *Eco*RI recognition site.

PCR amplification was carried out in a 100 µl reaction mixture containing the extracted bacterial nucleic acids (ca 10 ng), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each dNTP, 10 pmol each primer and 2.5 U Ampli *Taq* DNA Polymerase (Perkin-Elmer) with an automatic thermal cycler (Astec PC-700). The thermal profile involved 25 cycles of denaturation at 95°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 40 s. The results of amplification were analyzed by 1.5% agarose gel electrophoresis. The PCR products digested with *Bam*HI and *Eco*RI were purified by agarose gel electrophoresis using a GENECLEAN II kit (BIO 101) and were ligated into pBluescript (Stratagene) using a ligation kit (Takara) to transform *Escherichia coli* (DH5α). The plasmids containing inserts were purified by the alkaline lysis method and the polyethylene glycol (MW: 6000) precipitation method.

Table 1. Bacterial strains used in this study. All strains of *Vibrio penaeicida* were isolated from kuruma prawn. JCM: Japan Collection of Microorganisms; ATCC: American Type Culture Collection; NCMB: National Collection of Marine Bacteria; NCTC: National Collection of Type Cultures. [1]: type strain

Species	Strain (Source)
Vibrio penaeicida	KH-1[T] (= JCM 9123, Kagoshima Pref.)
	PD-A (= JCM 9124, Hiroshima Pref.)
	KA-13 (= JCM 9125, Yamaguchi Pref.)
	ESV-8701 (= JCM 9192, Ehime Pref.)
	PO-1 (= JCM 9193, Hiroshima Pref.)
	KO-1 (= JCM 9194, Oita Pref.)
	KT-1 (Yamaguchi Pref.)
V. alginolyticus	NCMB 1903[T]
V. anguillarum	ATCC 19264[T]
V. cholerae (non-O1)	PS-7701 (Ayu, Shiga Pref.)
V. damsela	ATCC 33539[T]
V. fluvialis	NCTC 11327[T]
V. harveyi	ATCC 14126[T]
V. nereis	ATCC 25917[T]
V. parahaemolyticus	ATCC 17802[T]
V. pelagius I	ATCC 25916[T]
V. proteolyticus	NCMB 1326[T]
V. splendidus II	ATCC 25914
V. tubiashii	ATCC 19109[T]
V. vulnificus II	ES-7601 (= ATCC 33147)
Escherichia coli	WP-2

The inserts in the plasmids were sequenced using a *Taq* Dye Primer Cycle Sequencing kit (ABI) and an automatic sequencer (ABI 373A). Several clones were used for determination of sequence to reduce the potential errors. The sequence data obtained were assembled and analyzed with DNASIS (Hitachi) and GENETYX (SDC). The sequence data for 16S rRNA of 9 *Vibrio* species including fish and shellfish pathogens (*Vibrio anguillarum*, *V. campbellii*, *V. diazotrophicus*, *V. hollisae*, *V. proteolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. parahaemolyticus*, and *V. cholerae*) were obtained from GenBank nucleotide sequence libraries and used for the comparative study.

RT-PCR amplification of 16S rRNA. A sense primer, 5'-GTGTGAAGTTAATAGCTTCATATC-3' (P-PJF primer), was designed based on the sequence of *Vibrio*

penaeicida 16S rRNA at the base positions 455 to 478. The R-GEN primer without the linker sequence was used as the reverse primer. The cDNAs to the 16S rRNA were synthesized in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM each dNTP, 10 pmol antisense primer, 1 U μl^{-1} RNase inhibitor (Toyobo), and 2.5 U μl^{-1} reverse transcriptase (M-MLV, USB) at 42°C for 30 min. PCR amplification of the cDNA was performed using the same procedure as described above except that the annealing temperature was 59°C, the final concentration of $MgCl_2$ was 2.5 mM, and the number of thermal cycles was 30.

RESULTS AND DISCUSSION

Kita-Tsukamoto et al. (1993) previously determined the 16S rRNA sequences of 50 strains of marine bacteria including 34 strains of the family Vibrionaceae, and showed that a region between base positions 440 and 490 of 16S rRNA was very variable among them. This was presumed to be true in *Vibrio penaeicida* as well. Thus, to amplify this variable region of *V. penaeicida*, R-GEN primer (786 to 805) and F-GEN primer (352 to 371) were designed as antisense and sense primers, respectively, from conserved regions flanking the variable region (Kita-Tsukamoto et al. 1993). In addition, the product expected to be amplified by these primers is of a suitable size (450 bp) for agarose gel electrophoresis analysis (Fig. 1).

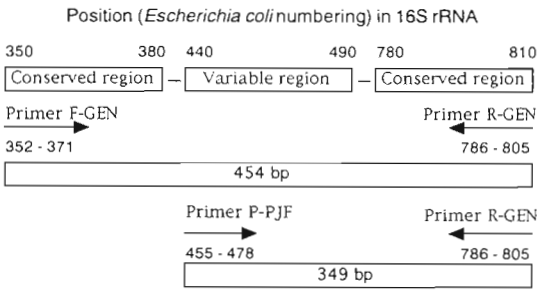

Fig. 1 Schematic illustration of the physical map of the PCR primers and 16S rRNA molecules of *Vibrio* species, showing variable and conserved regions according to Kita-Tsukamoto et al. (1993)

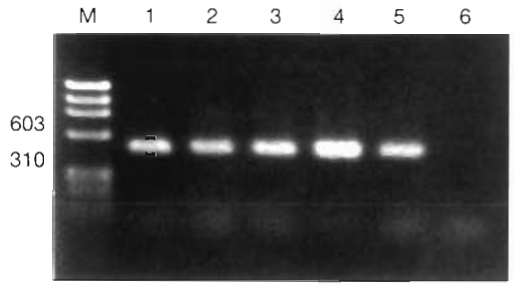

Fig. 2. Agarose gel electrophoretic analysis of the PCR products from nucleic acids of *Vibrio penaeicida* strains and other *Vibrio* species with the primers R-GEN and F-GEN. Lanes: M, length marker (ϕ X174/*Hae*III digest); 1, *V. penaeicida* KH-1; 2, KA-13; 3, KO-1; 4, *V. anguillarum*; 5, *V. harveyi*; 6, negative control (containing no template)

The target region of the rDNAs of 7 strains of *Vibrio penaeicida*, 4 other species of *Vibrio* (*V. anguillarum*, *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*), and *Escherichia coli* were all amplified by PCR using the R-GEN and F-GEN primers. Results of PCR in 3 strains of *V. penaeicida*, *V. anguillarum*, and *V. harveyi* are shown in Fig. 2. In the next step, the PCR products from 7 strains of *V. penaeicida* were cloned and sequenced. Multiple alignments of partial 16S rRNA sequences (352 to 589) of *V. penaeicida* and 9 fish- and

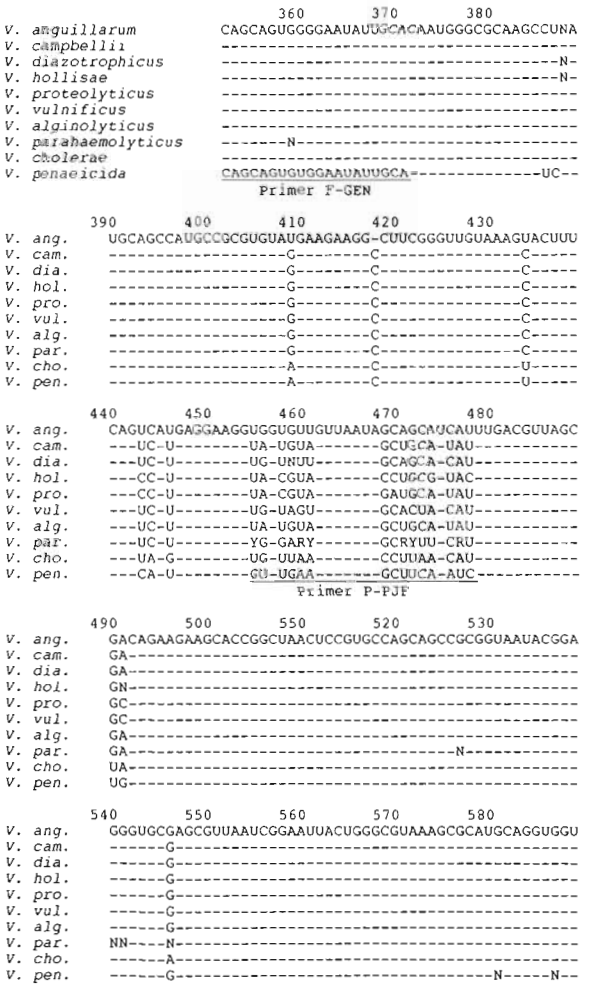

Fig. 3. Multiple alignments of the 16S rRNA sequences of *Vibrio penaeicida* and 9 other *Vibrio* species. Abbreviations for nucleotides used in the alignment: R = purine (A or G); Y = pyrimidine (C or U); N = A, C, G, or U

shellfish-pathogenic *Vibrio* species are shown in Fig. 3. There were no substitutions in the determined sequences of 16S rRNA among the 7 strains of *V. penaeicida*, while 18 substitutions were observed in the variable region (440 to 480) between *V. penaeicida* and others. Especially, 15 out of the 18 substitutions were concentrated between 455 and 478. Furthermore, no sequences having a high similarity with this region of *V. penaeicida* 16S rRNA were found in the GenBank data base.

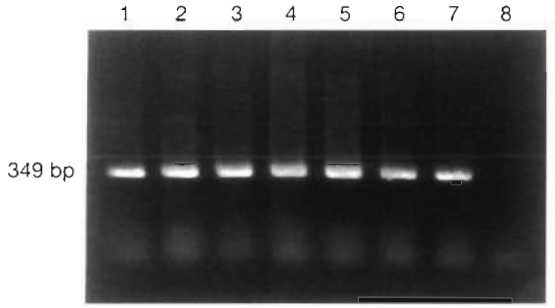

Fig. 4. Analysis of the PCR products from nucleic acids of 7 strains of *Vibrio penaeicida* with the primer P-PJF and primer R-GEN without linker sequence. Lanes: 1, KH-1; 2, KT-1; 3, KA-13; 4, PD-A; 5, ESV-8701; 6, KO-1; 7, PO-1; 8, negative control (containing no template)

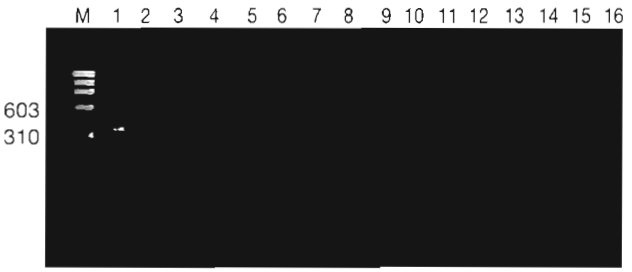

Fig. 5. Agarose gel electrophoretic analysis of the PCR products from nucleic acids of *Vibrio penaeicida* KH-1 and 13 other *Vibrio* species with the primer P-PJF and primer R-GEN without linker sequence. Lanes: M, length marker (ϕ X174/*Hae*III digest); 1, *V. penaeicida* KH-1; 2, *V. alginolyticus*; 3, *V. anguillarum*; 4, *V. cholerae* (non-O1); 5, *V. damsela*; 6, *V. fluvialis*; 7, *V. harveyi*; 8, *V. nereis*; 9, *V. parahaemolyticus*; 10, *V. pelagius* I; 11, *V. proteolyticus*; 12, *V. splendidus* II; 13, *V. tubiashii*; 14, *V. vulnificus* II; 15, *Escherichia coli*; 16, negative control (containing no template)

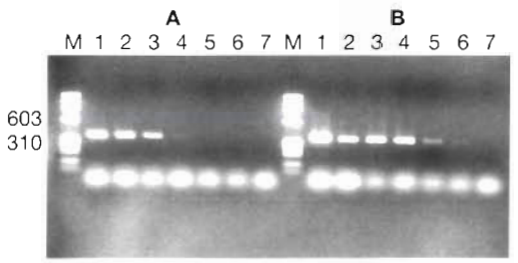

Fig. 6. Detection limit of purified nucleic acids from *Vibrio penaeicida* KH-1 by PCR and RT-PCR with the primer P-PJF and primer R-GEN without linker sequence. A: products by PCR; B: products by RT-PCR. Lanes: M, length marker (ϕ X174/*Hae*III digest); 1, 1 ng; 2, 100 pg; 3, 10 pg; 4, 1 pg; 5, 100 fg; 6, 10 fg; 7, 1 fg

An oligonucleotide identical to the species-specific sequence of *Vibrio penaeicida* 16S rRNA (455 to 478) was synthesized and used as a sense primer (P-PJF) to examine its specificity in PCR amplification. Although R-GEN primer without the linker sequence used as an antisense primer was 4 bases shorter than P-PJF, it had the same Tm as P-PJF because of its high GC%. A single PCR product with the expected length (350 bps) was amplified from the extracted nucleic acids of all 7 strains of *V. penaeicida* (Fig. 4), while no PCR products were obtained from those of 13 other *Vibrio* species or *Escherichia coli* (Fig. 5). The same results were observed using RT-PCR amplification (data not shown). These results indicate that the present PCR or RT-PCR amplification with P-PJF and R-GEN primers is useful for specific detection of *V. penaeicida*.

To compare the sensitivity of PCR and RT-PCR amplifications, serial 10-fold dilutions of nucleic acids extracted from *Vibrio penaeicida* strain KH-1 were prepared and tested. The PCR amplification needed at least 1 pg of the nucleic acids to yield a visible fragment on agarose gel electrophoresis, while in RT-PCR 10 fg of nucleic acids were sufficient to yield a visible fragment (Fig. 6). This higher sensitivity of RT-PCR compared with PCR can be explained by the fact that RT-PCR amplification could target both rDNA and rRNA.

In the present study, a species-specific sequence of *Vibrio penaeicida* 16S rRNA was demonstrated and RT-PCR amplification was developed as a specific and sensitive method for the detection of the pathogen. In applying the present RT-PCR procedure, an appropriate method for the extraction of bacterial nucleic acids should be developed to detect *V. penaeicida* from prawns where many kinds of inhibitors to PCR enzymes and/or RNase will be present.

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