Molecular identification of pathogenic *V. harveyi* and pathogenesis analysis

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Abstract

V. harveyi is the cause of serious disease in the shrimp industry in Thailand during cultivation. In this study, the *gyrB* gene of *V. harveyi* NICA, isolated from shrimp in Thailand, was sequenced. A pair of specific primers (A2B3) was designed that allowed amplification of a 363 bp gene fragment of *V. harveyi*. No cross reaction was detected in 17 other *Vibrio* species tested except for *V. carchariae* which is a synonym for *V. harveyi*. The possibility of using A2B3 for confirmation and enumeration of *V. harveyi* by PCR was demonstrated. Of 40 possible *V. harveyi* strains isolated from seafood on the basis of their growth on TCBS plates and biochemical reactions, 36 gave a reaction with the specific primers. The primers could detect *V. harveyi* at a level of as few as 15 cells/ml. The virulence factors of *Vibrio harveyi*, the causative agent of luminous vibriosis, are not completely understood. We investigated the correlations between shrimp mortality, hemolysis, the presence of a hemolysin gene (*vhh*), and a gene involved in the type III secretion system (the *Vibrio* calcium response gene *vcrD*). *V. harveyi* HY01 was isolated from a shrimp that died from vibriosis and 36 other *V. harveyi* isolates were obtained from fish and shellfish in Hat Yai city, Thailand. An ocean isolate of *V. harveyi* BAA-1116 was also included. Thirteen isolates including *V. harveyi* HY01 caused shrimp death 12 h after injection. Most *V. harveyi* isolates in this group (designated as Group A) caused hemolysis on prawn blood agar. None of the shrimp died after injection with *V. harveyi* BAA-1116. Molecular analysis of all *V. harveyi* isolates revealed the presence of *vcrD* in both pathogenic and non-pathogenic strains. Although *vhh* was detected in all *V. harveyi* isolates, some isolates did not cause hemolysis, indicating that *vhh* gene expression might be regulated. Analysis of the *V. harveyi* HY01 genome revealed a *V. cholerae* like-hemolysin gene, *hlyA* (designated as *hhl*). Specific primers designed for *hhl* detected this gene in 3 additional *V. harveyi* isolates but the presence of this gene was not correlated with pathogenicity. Random amplified polymorphic DNA (RAPD) analysis revealed a high degree of genetic diversity in all *V. harveyi* isolates, and there were no correlations among the *hhl*-positive isolates nor the pathogenic strains.

Introduction

Vibrio harveyi is a Gram-negative, luminous bacterium. It is widely distributed in aquatic environments and is considered to be an important causative agent of luminous disease in marine organisms. Over the past decade, strains of this species have been reported to be significant pathogenic agents and one cause of the high rates of shrimp mortality in the shrimp culture industry worldwide (Karunasagar et al., 1994; Saeed, 1995; Liu et al., 1996a; Liu et al., 1996b). Mortalities of *Penaeus monodon* and *P. merguiensis* larvae associated with luminescence have been observed in hatcheries in Indonesia (Sunaryanto and Marium, 1986), Phillippines (Lavilla-Pitogo et al., 1990; Baticados et al., 1991) and Taiwan (Chen et al., 1992; Song and Lee, 1993). In Thailand, *V. harveyi* has been reported to cause 70–100% of deaths in *P. merguiensis* larvae at the nauplii, mysis and postlarva stage with nauplii larvae being the most sensitive (Sae-Oui et al., 1987). In southern parts of Thailand, *V. harveyi* is the most important pathogen of the black tiger shrimp, *P. monodon,* in shrimp farms (Ruangsri et al., 2004). Raungpan et al. (1995) also found that when *Vibrio* and luminous bacteria exceeded 10^4 cells/ml in overcrowded cultured shrimp ponds, this caused serious health problems to the shrimp. Vibriosis in cultured shrimps causes severe economic losses in shrimp production. A method for the early detection of *V. harveyi* contamination or infection could facilitate disease prevention in the shrimp aquaculture industry. Because of the very close phylogenetic relationship of this organism to other *Vibrio* species such as *V. parahaemolyticus, V. alginolyticus, V. campbellii* and *V. carchariae* (Kita-Tsukamoto et al., 1993; Pedersen et al., 1998), identification of *V. harveyi* by conventional biochemical techniques is not accurate. Therefore, a PCR method that could target nucleotide sequences unique to *V. harveyi* may facilitate its detection and differentiation from closely related *Vibrio* species. In addition, it may be useful for the enumeration of *V. harveyi.*

The gene *gyrB* encodes for the subunit B protein of DNA gyrase (topoisomerase type II). DNA gyrase regulates the supercoiling of double-stranded DNA. It is necessary for DNA replication, and the enzyme is distributed universally among bacterial species (McMacken et al., 1987). In addition, its molecular evolution rate is higher than that of 16S rRNA. This divergence in the *gyrB* gene could provide greater resolution for phylogenetic analysis of luminous bacteria than does the 16S rRNA method (Dunlap and Jennifer, 2005) and *gyrB* targeted PCR protocols have been used for identification of other bacteria such as *Aeromonas, Pseudomonas, Bacillus*, and *V. hollisae* (Yamamoto and Harayama, 1995; Vuddhakul et al., 2000; Yamamoto et al., 2000; Yanez et al., 2003; De Clerck et al., 2004). Therefore, in this study, PCR primers that could amplify the *gyrB* gene of *V. harveyi* were developed to test their specificity for the identification and enumeration of *V. harveyi* in postlarva shrimp and hatchery tank water.

Many bacteria of the genus *Vibrio*, including *V. cholerae, V. parahaemolyticus, V. vulnificus, V. anguillarum*, and *V. mimicus*, possess hemolysins (Zhang & Austin 2005). Most of these hemolysins have been reported to be important virulence factors and some hemolysin genes have only been detected in pathogenic strains (Miyamoto et al. 1969, Honda et al. 1988). *V. harveyi* is a halophilic bacterium widely distributed in marine environments. It is a causative agent of luminous vibriosis and causes major economic losses in the shrimp farming industry worldwide

(Lavilla-Pitogo et al. 1990, Karunasagar et al. 1994, Saeed 1995). Differences in pathogenicity between isolates of *V. harveyi* have been reported, and the role of hemolysins in the virulence of *V. harveyi* has not been fully determined. *V. harveyi* isolated from diseased penaeids were more virulent to tiger prawns and showed higher hemolytic activities against sheep and fish erythrocytes compared with non-virulent isolates from sea water or diseased *Talorchestia* sp. (Liu et al. 1996). Investigations of the pathogenicity of *V. harveyi* isolates in fish (Atlantic salmon and rainbow trout) have demonstrated that both pathogenic and non-pathogenic *V. harveyi* isolates induced hemolysis against erythrocytes from sheep, rabbit, donkey, and horse, and the presence of the hemolysin gene *vhh* has been demonstrated in *V. harveyi* (Zhang & Austin 2000, Zhang et al. 2001). An investigation of the mortality of Artemia franciscana nauplii after inoculation with *V. harveyi* isolates from healthy and diseased penaeid shrimp from Asia and the Americas indicated that particular exoenzymes were associated with virulent strains (Soto-Rodriguez et al. 2003). No correlation between the hemolytic activity against sheep erythrocytes and the death of infected shrimps was detected (Soto-Rodriguez et al. 2003). Further research is needed to resolve these controversies between the pathogenicity of *V. harveyi* and its ability to cause hemolysis.

Recent studies have shown that many bacteria use a cell–cell communication process known as quorum sensing to control cell population density and ensure that a sufficient number of bacteria are present to coordinate a virulence response that will overwhelm host defenses (Miller & Bassler 2001, Winzer & Williams 2001). Quorum sensing has a role in bacterial pathogenicity as it regulates the secretion of virulence factors and biofilm formation (Miller & Bassler 2001, Donabedian 2003). Quorum sensing has been reported in *Vibrio fisheri*, *V. cholerae*, *V. parahaemolyticus*, and *V. harveyi* (Stevens & Greenberg 1997, Cámara et al. 2002, Henke & Bassler 2004a). The virulence of *V. harveyi* may be controlled by a quorum sensing mechanism involving the type III secretion system (TTSS) (Henke & Bassler 2004a). TTSS is a bacterial system that transfers effector virulence proteins across the membrane of the bacterial pathogen into the cytoplasm of the host cell and has a crucial role in host–pathogen interactions. TTSS is found in many Gram-negative bacteria including *V. parahaemolyticus*, *V. alginolyticus*, *V. cholerae*, and *V. harveyi* (Makino et al. 2003, Park et al. 2004, Dziejman et al. 2005). The TTSS gene cluster in *V. harveyi* includes *vop*, *vsc*, and *vcr*, which encode a *Vibrio* outer membrane protein, *Vibrio* secretion protein, and *Vibrio* calcium response protein, respectively (Henke & Bassler 2004b). *vcrD* is homologous to the low calcium response gene *lcrD*, a conserved gene encoding an essential component of the secretion apparatus in *Yersinia* spp., and *lcrD* homologs are present in all known TTSSs (Hueck 1998). In the present study, we investigated the correlations between *V. harveyi* pathogenicity in a shrimp model, hemolysis, hemolysin genes, and *vcrD*, a gene involved in the TTSS of *V. harveyi*.

Material and Methods

Bacterial strains, growth conditions and DNA extraction

Vibrio harveyi NICA was obtained from the National Institute of Coastal Aquaculture (NICA). Others (listed in Table 2) were from our laboratory stock strains or strains supplied by other workers for this study. *V. harveyi* was grown overnight, at 25°C, with shaking in Luria Bertani (LB) broth containing 1% NaCl. Chromosomal DNA was extracted with phenol–chloroform (Sambrook et al., 1989).

Use of degenerate PCR primers to amplify the *V. harveyi gyrB* gene

The degenerate primers UP-1 and UP-2r designed by Yamamoto and Harayama (1995) were used in this study to amplify a *gyrB* homologue in *V. harveyi* NICA strain. Using PCR to amplify this gene, the 20-μl reaction mixture contained 6 μl of purified cellular DNA of *V. harveyi* (0.175 μg/ml), 2 μl of each of the PCR primers (10 μM), 2 μl of 10 x buffer containing 20 mM $MgCl_2$ (ExTag buffer; Takara, Shiga, Japan), 1 μl (0.5 U) of *Tag* polymerase (ExTag; Takara), 2 μl of each deoxynucleotide triphosphate (2.5 mM), and 5 μl of distilled water. The amplification conditions were 30 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min, followed by 1 cycle of 72°C for 7 min. Amplification was performed in a Perkin-Elmer Thermal Cycler (GeneAmp PCR System 2400).

Cloning sequencing and designing primers

The amplicon of 1.3 kb obtained from the PCR method described in section 2.2 was purified using a gel purification kit (QIAGEN, Germany) and subsequently cloned into pGEM-T Easy (Promega Corp., Madison, WI), using *Escherichia coli* MC1061 as the host. The presence of the expected size inserts within the plasmids of transformants was verified by restriction enzyme analysis. The nucleotide sequences of both strands of the cloned fragment were determined with the ABI-PRISM 310 genetic analyzer (Applied Biosystems Division, Perkin-Elmer, Foster City, CA). The sequence of the partial *gyrB* of *V. harveyi* is deposited in the GenBank database under accession number DQ345719. The homology search sequence of the *gyrB* gene of *V. harveyi* compared to other *Vibrio* spp. was performed using Blast. For designing primers, the nucleotide sequence of part of the *V. harveyi gyrB* was compared with the *gyrB* from other *Vibrio* species using the ClustalW program. The regions that were not well conserved between the *V. harveyi* sequence and the sequences of the other *Vibrio* spp. were selected (Fig. 1). The *V. harveyi gyrB* sequences of these regions were examined with a computer program for designing PCR primers (Oligo 4.0, National Bioscience, Inc., Plymouth, MN). Pairs of three primers thus designed were evaluated (Table 1).

V. harveyi gyrB-specific PCR

V. harveyi NICA, *V. harveyi* ATCC14126, *V. harveyi* PSU 42, *V. harveyi* PSU 43, *V. harveyi* PSU 45, *V. harveyi* PSU 2529, *V. harveyi* PSU 2530, *V. fluvialis, V. furnisii, V. mimicus, V. vulnificus, V. campbellii, V. carchariae, V. mytili, V. hollisae*

(Grimontia hollisae), V. splendidus, V. metschnikovii, V. orientalis, V. anguillarum, V. alginolyticus, V. proteolyticus, V. cholerae O1, *V. cholerae* O139, *V. cholerae* non-O1, non-O139, *V. parahaemolyticus, Photobacterium damselae, Aeromonas hydrophila, Escherichia coli, Shigella flexneri* and *Shigella boydii* (Table 2) were grown overnight in LB broth containing 1% NaCl. One milliliter of the broth culture was boiled for 10 min, and the supernatant was obtained by centrifugation and diluted 10-fold in distilled water. The diluted supernatant was used as the template for PCR amplification.

The PCR mixture consisted of 2 μl of DNA template, 1 μl of 2.5 mM deoxyribonucleotide triphosphate, 5 μl of each of the primers (2 μM), 0.1 μl of *Tag* polymerase (*Tag* DNA polymerase in storage buffer A (5 U/μl) Promega Corp.), 2 μl of 10 x buffer (thermophilic DNA polymerase 10 x buffer, magnesium free; Promega Corp.), 1.2 μl of 25 mM $MgCl_2$ and 3.7 μl of distilled water. The amplification conditions were 30 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 2 min, followed by 1 cycle of 72°C for 7 min. Amplification was performed in a Perkin-Elmer Thermal Cycler (Gene Amp PCR System 2400). Ten microliters of PCR product was resolved by electrophoresis in 1.5% agarose gel to detect amplicons of the expected size.

Sensitivity of the primers

V. harveyi NICA was cultured overnight on LB agar at room temperature and then inoculated into LB broth and incubated at room temperature for 3 h. The concentration of cells was adjusted to 1.5×10^8 cells/ml using the 0.5 McFarland Standard, then serially diluted 10-fold from 1.5×10^8 to 1.5 cells/ml. One milliliter of each dilution was boiled for 10 min, the DNA template was prepared and PCR was performed to determine the sensitivity of the primers as previously described.

Identification of *V. harveyi* isolated from seafood

One hundred and twenty samples of seafood including shrimp, fish, mollusks, mantis-shrimp and crab were investigated. *V. harveyi* was isolated from shrimp. Briefly, small shrimp were crushed in a sterile mortar. For adult shrimp, *V. harveyi* was isolated from the hemolymph and hepatopancreas and was isolated from skin, gills and intestine from fish. In shellfish, it was isolated from the hemolymph. In all cases, sucrose non-fermenting colonies on thiosulfate-citrate-bile salts-sucrose agar (TCBS) were selected after incubation at room temperature for 24 h and identified as *V. harveyi* by biochemical tests following the schemes of Alsina and Blanch (1994) and confirmed by the PCR method described in section 2.4.

Bacterial isolates

Vibrio harveyi HY01 was isolated from a black tiger shrimp that died from luminous vibriosis in Hat Yai city, Thailand. A total of 36 *V. harveyi* isolates were

obtained from fish, shrimp, and mollusks in the same city (Thaithongnum et al. 2006). These isolates were identified by biochemical testing and confirmed by PCR targeted to the DNA gyrase subunit B (*gyrB*) gene. An ocean isolate of *V. harveyi* BAA-1116 (BB210) (Bassler et al. 1997) was provided by Prof. John Mekalanos, Department of Microbiology and Molecular Genetics, Harvard University, USA. The standard strain of *V. harveyi* was obtained from the National Institute of Coastal Aquaculture (NICA), Thailand. *V. carchariae*, *V. cholerae* O1, *V. cholerae* non-O1, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. campbellii*, *V. furnissii*, *V. fluvialis*, *V. hollisae* (*Grimontia hollisae*) and *Escherichia coli* were from our laboratory stock strains. All bacterial strains were kept at -80°C and were grown overnight on Luria-Bertani (LB) agar containing 1% NaCl at 30°C before being assayed.

Virulence investigation *in vivo*

The median lethal dose (LD_{50}) of *Vibrio harveyi* HY01 pathogenic strain and an ocean isolate of *V. harveyi* BAA-1116 was evaluated on the black tiger shrimp *Penaeus monodon*. The test strains were grown overnight in tryptic soy broth containing 1% NaCl at 30°C with shaking at 150 rpm. Cultured cells were harvested by centrifugation at 200 × *g* for 10 min, and washed twice with sterile artificial sea water (ASW) (Marinum). Bacterial cell suspensions in ASW were adjusted to 2.5×10^8 colony-forming units (CFU) ml^{-1} using a turbiditometer (Oxoid) and 2-fold dilutions were performed to obtain concentrations of bacteria between 4.0×10^5 and 6.4×10^6 CFU.

The *Penaeus monodon* juvenile shrimps used in the present study were 10 to 13 g in weight and 4 to 5 inches (10.16 to 12.7 cm) in length. Each shrimp received an intramuscular injection of 0.1 ml diluted *Vibrio harveyi* (with batches of 7 shrimps $dose^{-1}$) between the third and fourth abdominal segments. Control shrimps were injected with ASW. The experiments were performed in duplicate. Shrimps were maintained in a 70 l ASW glass tank at a temperature of 29 ± 1°C and salinity of 17 ppt. Shrimp mortalities were observed within 48 h of injection and were confirmed by detecting luminescence in the organs of the dead shrimp. The LD_{50} of *V. harveyi* was calculated using the method of Reed & Muench (1938).

For evaluation of virulence of all *Vibrio harveyi* isolates obtained from fish and shellfish, each isolate was injected into shrimps at a concentration of 4× LD_{50} (Kashef et al. 2006), using the same procedure as described in the previous paragraph. Mortality rates were measured 48 h after injection.

Hemolysis detection

Prawn blood agar was prepared according to the technique of Chang et al. (2000). Briefly, 1 ml of citrate-EDTA prawn hemolymph was mixed with Rose Bengal (at a final concentration of 0.3% w/v) and was added to 15 ml basal medium (consisting of 10 g Bacto peptone, 10 g NaCl, and 15 g Bacto agar in 1 l). Hemolysis was evaluated by detection of a clear zone around *Vibrio harveyi* colonies on prawn blood agar within 7 d of incubation at 30°C.

Testing for hemolysin and TTSS genes

vhh-F and *vhh*-R primers specific to the *vhh* hemolysin gene of *Vibrio harveyi* were designed using *vhh* sequences of *V. harveyi* VIB 645 (accession nos. AF 293430 and AF 293431) and *V. harveyi* BAA-1116 (accession no. NC_009784) reported in GenBank. *vcrD1* and *vcrD2* primers targeted to the *vcrD* gene, a gene of the TTSS of *V. harveyi*, were designed based on the *vcrD* nucleotide sequences of *V. parahaemolyticus* (accession no. NP_798041), *V. alginolyticus* (accession no. ZP_01259742), and *V. campbellii* (accession no. ZP_02195895) deposited in GenBank. All primers were designed using MacVector 9.5.2 and BLAST software (www.ncbi.nlm.nih.gov/BLAST/), and are shown in Table 4. To evaluate the specificity of these primers, *V. harveyi* NICA, *V. harveyi* HY01, *V. carchariae*, *V. cholerae* O1, *V. cholerae* non-O1, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. campbellii*, *V. furnissii*, *V. fluvialis*, *V. hollisae*, and *Escherichia coli* were grown overnight in LB broth containing 1% NaCl, and DNA templates were extracted by boiling (Thaithongnum et al. 2006). PCR analysis was performed in 20 µl of reaction mixture containing 2 µl of 10× PCR buffer (Promega), 1.5 µl of template DNA, 1.6 µl of 25 mM $MgCl_2$, 5 µl of *vhh*-F and *vhh*-R or *vcrD1* and *vcrD2* primers (2 µM), 0.1 µl of *Tag* polymerase (Promega), and 1.6 µl of 2.5 mM dNTPs. PCR analysis was performed using a Gene Amp PCR thermocycler, and the reaction involved 96°C for 5 min, 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, followed by 72°C for 7 min. The amplification products were subjected to electrophoresis in 1% agarose gel.

A total of 38 isolates of *Vibrio harveyi* were examined for the presence of *vhh* and *vcrD*. Southern-blot hybridization assays were used to confirm isolates that were PCR-negative for *vhh* (Bhoopong et al. 2007). Briefly, genomic DNA from *V. harveyi* was digested with *Eco*RI restriction enzyme, the *vhh* probe was prepared by PCR using the *vhh* primers designed in the previous paragraph, and the probe was labeled with digoxigenin. Hybridization was performed under high-stringency conditions at 42°C. The hybridized probes were detected using a DNA detection kit (Roche Diagnostics) according to the manufacturer's instructions.

hlyA-like hemolysin gene

Using BLAST software, a sequence alignment of the hemolysin gene from *Vibrio harveyi* strain HY01 (GenBank accession no. NZ_AAWP01000001) showed 86% homology to the *hlyA* hemolysin gene of *V. cholerae* N16961 (GenBank accession no. AE003853). Therefore, specific primers for this gene, designated as *hhl*, were designed (Table 4). Amplification conditions for this gene were similar to those used for *vcrD*, except that 1 µl of each primer (2 µM concentration) was used and the PCR reaction comprised 94°C for 5 min, 30 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 1 min. The presence of this *hhl* gene was examined in *V. harveyi* HY01 and *V. harveyi* BAA-1116, and 36 isolates of *V. harveyi* from fish and shellfish. Some *hhl*-negative isolates were confirmed by Southern-blot hybridization using the *hhl* probe.

Random amplified polymorphic DNA (RAPD) fingerprinting

To characterize DNA profiles of pathogenic and non-pathogenic strains of *Vibrio harveyi*, genomic DNA of *V. harveyi* was extracted by a phenol-chloroform method and RAPD was performed using the RAPD universal primer 2 (5′-GTT TCG CTC C-3′) (Radu et al. 1998, Wootipoom et al. 2007). Amplification was performed in a 30 µl mixture containing 0.33 mM dNTPs (TaKaRa Biochemicals), 25 ng of template DNA, 2.5 U *Ex Taq* (TaKaRa), 0.83 pmol of primer, and 1× buffer. The PCR analysis was performed in an Astec PC-818A thermal cycler and the reaction comprised 95°C for 4 min, 45 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min, followed by 72°C for 7 min. The amplification products were analyzed by electrophoresis using a 1.5% agarose gel.

Using a Bioprofile image analysis system (Viber Lourmat), a dendrogram was constructed using the data matrix generated by the DNA profiles of all 38 tested strains of *Vibrio harveyi* (Sneath & Sokal 1973).

Statistical analysis

Pearson's correlation coefficient was used to investigate correlations between hemolytic activity and shrimp mortality (Neter et al. 1996).

Results

gyrB sequence of *V. harveyi*

The amplified sequence of the *gyrB* gene of *V. harveyi* NICA was 1,174 bp long. The homology of this gene sequence compared to the partial gene sequence of *V. harveyi gyrB* (AF007289) in GenBank (420 bp) was 93% and showed 87% homology with the *V. parahaemolyticus gyrB* sequence. Therefore, this sequence is in the expected region of the *V. harveyi gyrB* gene.

V. harveyi specific primers

Three pairs of PCR primers were designed, targeted to the internal fragments of *V. harveyi* NICA *gyrB* gene and these produced a product size of between 337 and 502 bp (Table 1). They were tested for the ability to amplify the *gyrB* gene of *V. harveyi* NICA, *V. harveyi* ATCC 14126, five isolates of *V. harveyi*, 18 other *Vibrio* species and 4 genera of non-vibrio, *Photobacterium*, *Aeromonas, Escherichia* and *Shigella* (Table 2). Primers A2B3 (Fig. 1) were the only primers that were specific for *V. harveyi* and its synonym, *V. carchariae*, and gave an amplicon of 363 bp. These primers could detect *V. harveyi* present in numbers as low as 1.5 × 10 cells/ml (Fig. 2).

Identification of *V. harveyi* in seafood

Eighty-one strains of bacteria were selected from TCBS plates from 120 samples of seafood (Table 3). Forty of these strains were suspected to be *V. harveyi* by biochemical tests but only 36 strains proved to be positive after confirmation by PCR using the A2B3 primers.

Table 1 Primer pairs tested for identification of *V. harveyi* NICA

Primers	Primers sequence 5' ⟶ 3'	Annealing site	Product size (bp)
1. A1	TTCATACGCAAACTTACCATC	105–441	337
B3	AGCAATGCCATCTTCACGTTC		
2. A2	TCTAACTATCCACCGCGG	79–441	363
B3	AGCAATGCCATCTTCACGTTC		
3. F1	GGTGTTTCTATCAAGCTGGT	281–792	502
B5	GCTTCTGTCGGGTTCTCAATC		

Table 2 Identification of *V. harveyi* and *Vibrio* species using designed primers

No.	Bacteria	A1B3	A2B3	F1B5
1	*Vibrio harveyi* NICA	+	+	+
2	*Vibrio harveyi* ATCC 14126	+	+	+
3	*Vibrio harveyi* PSU 42	+	+	+
4	*Vibrio harveyi* PSU 43	+	+	+
5	*Vibrio harveyi* PSU 45	+	+	+
6	*Vibrio harveyi* PSU 2529	+	+	+
7	*Vibrio harveyi* PSU 2530	+	+	+
8	*Vibrio fluvialis* PSU 90	–	–	–
9	*Vibrio furnisii* PSU 101	–	–	–
10	*Vibrio mimicus* PSU 78	–	–	–
11	*Vibrio vulnificus* PSU 89	–	–	–
12	*Vibrio campbellii* ATCC 25920	–	–	–
13	*Vibrio carchariae* ATCC 35084	+	+	+
14	*Vibrio hollisae* PSU 39	+	–	+
15	*Vibrio splendidus* ATCC 33125	–	–	–
16	*Vibrio mytili* ATCC 51288	–	–	–
17	*Vibrio metschnikovii* PSU 121	–	–	–
18	*Vibrio orientalis* ATCC 33934	–	–	–
19	*Vibrio anguillarum* PSU 56	–	–	–
20	*Vibrio alginolyticus* PSU 67	–	–	–
21	*Vibrio proteolyticus* PSU 254	–	–	–
22	*Vibrio cholerae* O1 PSU 368	–	–	–
23	*Vibrio cholerae* O139 PSU 450	–	–	–
24	*Vibrio cholerae* non-O1, non-O139 PSU 449	–	–	–
25	*Vibrio parahaemolyticus* PSU 241	–	–	–
26	Photobacterium damselae PSU 110	–	–	–
27	*Aeromonas hydrophila* PSU 376	–	–	–
28	*Escherichia coli* PSU 165	–	–	–
29	*Shigella flexneri* PSU 99	–	–	–
30	*Shigella boydii* PSU 365	–	–	–

Table 3 Identification of *V. harveyi* isolated from seafood using phenotypic and PCR targeted to the *gyrB*

Seafood	Total no. of samples	No. of bacteria isolated from TCBS plates	*V. harveyi* (biochemical test)	*V. harveyi* (PCR)
Shrimp	74	54	28	27
Fish	22	17	8	6
Mollusks	20	9	4	3
Mantis-shrimp	2	1	0	0
Crab	2	0	0	0
Total	120	81	40	36

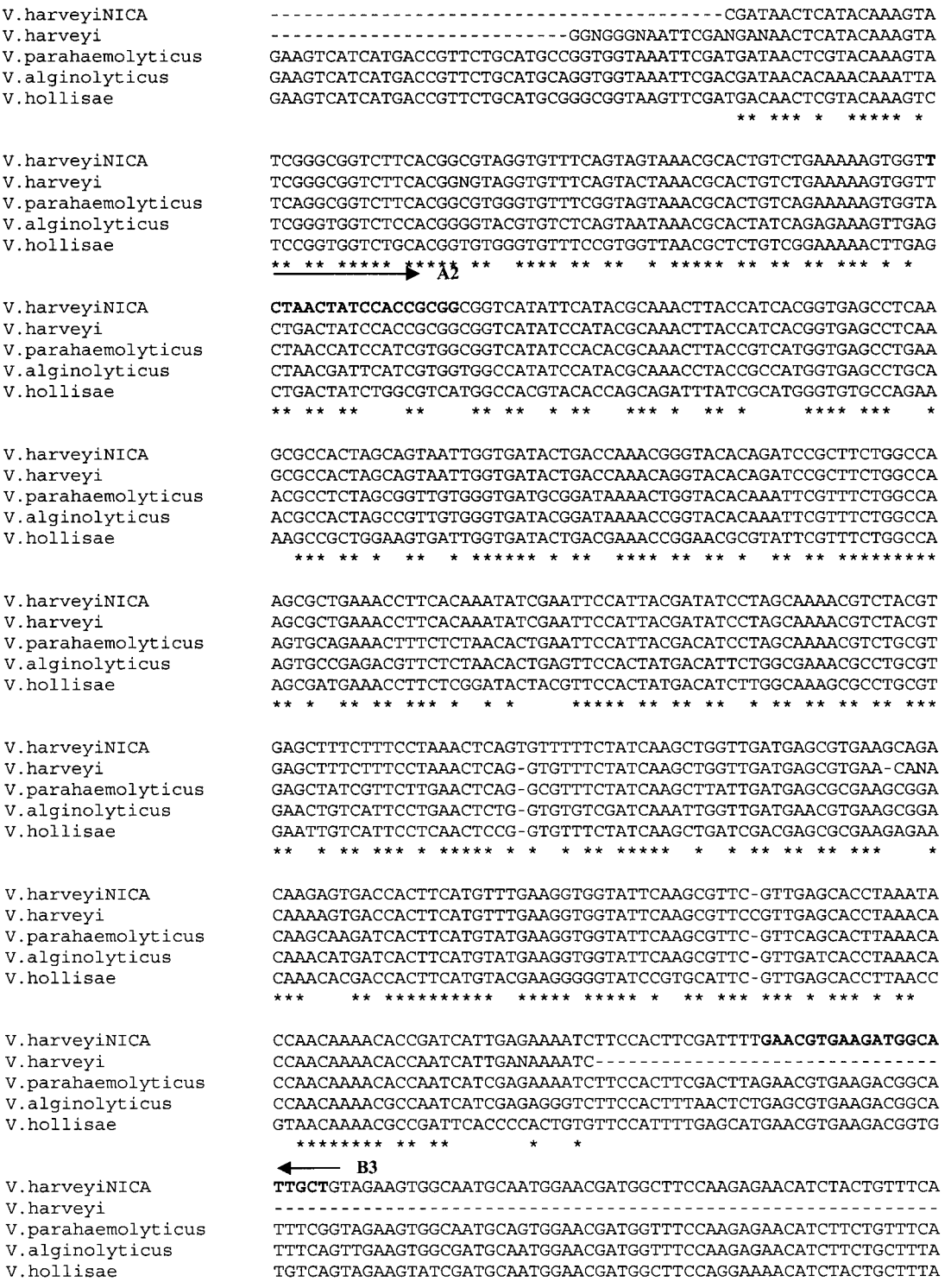

Fig. 1. Multiple alignment of *gyrB* sequences of *V. harveyi* NICA and four other *Vibrio* species showing non-identical residues at A2B3 specific primers. *V. harveyi* NICA, *V. harveyi* AF007289, *V. parahaemolyticus* AF007287, *V. alginolyticus* AF 007288 and *V. hollisae* AB 027462

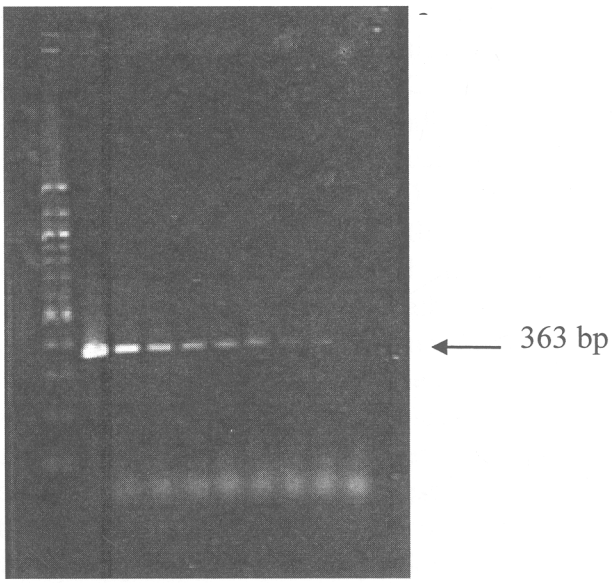

Fig. 2. Sensitivity of the A2B3 primers for detection of *V. harveyi*
M = MW marker, Lanes 1–9 = *V. harveyi* 1.5×10^{8}, 1.5×10^{7}, 1.5×10^{6}, 1.5×10^{5}, 1.5×10^{4}, 1.5×10^{3}, 1.5×10^{2}, 1.5×10 and 1.5 cells/ml, respectively

Determination of shrimp pathogenicity

The LD_{50} of *Vibrio harveyi* was evaluated using *V. harveyi* strains HY01 and BAA-1116. Mortality rates of 85.7 and 58.3% were observed in injected shrimp after injection of *V. harveyi* HY01 at concentrations of 6.4×10^6 and 3.2×10^6 CFU shrimp^{-1}, respectively (Table 5). No shrimp deaths were detected after injection of *V. harveyi* BAA-1116. Therefore, the LD_{50} of *V. harveyi* HY01 was calculated to be 1.9×10^6 CFU shrimp^{-1}. Inoculum concentrations equivalent to $4\times$ LD_{50} were used to analyze the pathogenicity of 38 *V. harveyi* isolates. We classified the *V. harveyi* isolates into 5 groups (A to E) according to their ability to cause shrimp death within a certain time period post-injection, with Group A being the most pathogenic and Group E the least. Thirteen isolates of *V. harveyi* were classified in Group A, including *V. harveyi* HY01 (Fig. 3, Table 6), because they were the most pathogenic strains, inducing 100% mortality within 12 h of injection. No live shrimp were detected at 24 h after injection with isolates of Group B (11 isolates). *V. harveyi* isolates of Group C (9 isolates) induced 58.0% mortality among test shrimp by 24 h after injection, with no subsequent deaths. The isolates designated as belonging to Group D (4 isolates) caused 29.0% mortality within 24 h of injection with no subsequent mortality thereafter. None of the shrimp died after injection with *V. harveyi* BAA-1116, which was designated as Group E.

Detection of hemolysis

Hemolysis on prawn blood agar was evaluated for all *Vibrio harveyi* isolates. It is of interest that 12 of the 13 *V. harveyi* isolates (92.3%) in Group A, including *V. harveyi* HY01, caused hemolysis, whereas the hemolytic activity of isolates of Groups B, C, and D was 81.8, 66.7, and 50.0%, respectively (Table 6). No hemolysis was observed on prawn blood agar inoculated with *V. harveyi* BAA-1116.

Detection of *vhh* and *vcrD* genes

The specificities of the *vhh* and *vcrD* primers (Table 4) were evaluated using 2 strains of *Vibrio harveyi*, 9 species of *Vibrio*, and *Escherichia coli*. Only *V. harveyi* produced an amplicon with the *vhh* primers (Fig. 4). For the *vcrD* primers, only *V. harveyi*, *V. carchariae*, and *V. parahaemolyticus* gave a positive result with a single 332 bp DNA product. This result was not surprising because previous analysis of 16S ribosomal DNA sequences has indicated that *V. carchariae* is the junior synonym of *V. harveyi* (Gauger & Gómez-Chiarri 2002), and the sequence, organization, and regulation of the TTSS gene clusters in *V. harveyi* and *V. parahaemolyticus* have been reported to be similar (Henke & Bassler 2004a).

All 38 isolates of *Vibrio harveyi* were analyzed for the presence of the *vhh* and *vcrD* genes using PCR. *vcrD* was detected in 35 *V. harveyi* isolates (91.7%), but not among

isolate numbers 1, 2, and 18 (Table 6). *vhh* was detected in 34 isolates (89.5%) and negative in the remaining 4 isolates (isolate numbers 1, 18, 8, and 24).

Detection of the *hhl* gene

The *hhl* gene was detected in 3 isolates of *Vibrio harveyi* (isolate numbers 15, 5, and 11) obtained from fish and shellfish (Table 6). These 3 isolates and *V. harveyi* HY01 contained both *vhh* and *hhl* hemolysin genes. Interestingly, 3 isolates that were negative for both *vhh* and *hhl* (isolate numbers 1, 18, and 8) showed hemolytic activity against prawn erythrocytes (Table 6). Therefore, Southern-blot hybridization was performed using the *vhh* and *hhl* probes (Fig. 5), and these isolates (plus isolate number 24) were shown to be *vhh*-positive (Fig. 5a).

RAPD fingerprinting

DNA fingerprinting of all *Vibrio harveyi* isolates was performed using the RAPD technique. Analysis of DNA profiles revealed 1 to 27 amplicons ranging from 300 to 12000 bp. Although all of the isolates shared one 1600 bp amplicon, 16 distinctive DNA profiles at 70% similarity were observed (Figs.6 & 7). *V. harveyi* HY01 and all 3 *V. harveyi* isolates with *hhl* showed different DNA profiles.

Table 4. Primers used for detecting *vhh*, *vcrD*, and *hhl* genes in *Vibrio harveyi*. W = A or T; R = A or G

Primer	Primer sequence 5′→3′	Position	Product size (bp)
vhh-F	AAGTAATCAGCAGCAGACGAGCG	3006–3919	914
vhh-R	GAGTGGGCAGAAAATCCAGATGG	1333–1664	332
vcrD1	TGWRACACGGGTAACGATGA	2287–3305	1019
vcrD2	GTAAGCAGATGAGRATCGACGG		
hhl-F	TTCAGAAAGACTTATGGGCTGGG		
hhl-R	TAAACACCGTGATAGATTGGGCG		

Table 5. Investigation of the median lethal dose (LD_{50}) of *Vibrio harveyi* strains HY01 and BAA-1116 in *Penaeus monodon*. The experiments were performed in duplicate and % mortality of shrimps in both experiments was not different. Therefore, the results of 1 experiment are shown here. CFU = colony-forming unit

V. harveyi strain	Dose (CFU shrimp^{-1})	No. of shrimp dead/total	Cumulative no. of shrimp		Mortality (%)
			Dead	Alive	
HY01	6.4×10^6	5/7	12	2	85.7
	3.2×10^6	4/7	7	5	58.3
	1.6×10^6	3/7	3	9	25.0
	8.0×10^5	0/7	0	16	0
	4.0×10^5	0/7	0	23	0
BAA-1116	6.4×10^6	0/7	0	7	0
	3.2×10^6	0/7	0	7	0
	1.6×10^6	0/7	0	7	0
	8.0×10^5	0/7	0	7	0
	4.0×10^5	0/7	0	7	0

Table 6. Detection of hemolysis and of *vcrD*, *vhh*, and *hhl* genes in *Vibrio harveyi* isolates

Isolate no.	Hemolysis on prawn blood agar	PCR detection of			Group
		vcrD	*vhh*	*hhl*	
4	+	+	+	–	A
7	+	+	+	–	A
23	+	+	+	–	A
25	+	+	+	–	A
26	+	+	+	–	A
30	+	+	+	–	A
33	+	+	+	–	A
34	+	+	+	–	A
9	–[a]	+	+	–	A
1	+	–	–	–	A
2	+	–	+	–	A
18	+	–	–	–	A
8	+	+	–	–	B
19	+	+	+	–	B
20	+	+	+	–	B
21	+	+	+	–	B
27	+	+	+	–	B
31	+	+	+	–	B
32	+	+	+	–	B
36	+	+	+	–	B
15	–	+	+	+	B
17	+	+	+	–	B
14	–	+	+	–	B
16	+	+	+	–	C
22	+	+	+	–	C
29	+	+	+	–	C
12	–	+	+	–	C
13	–	+	+	–	C
24	–	+	–	–	C
6	+	+	+	–	C
28	+	+	+	–	C
35	+	+	+	–	C
5	+	+	+	+	D
3	+	+	+	–	D
10	–	+	+	–	D
11	–	+	+	+	D
HY01	+	+	+	+	A
BAA-1116	–[b]	+	+	–	E

[a]Not detected

[b]Not observed due to poor growth compared with other strains tested on the same prawn blood agar plates after 7 d of incubation

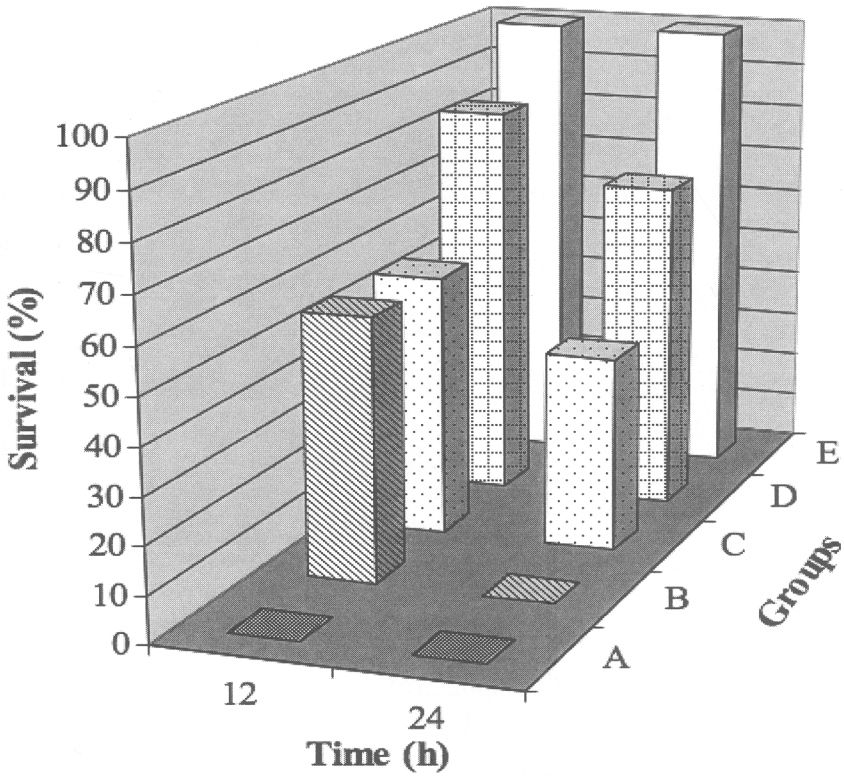

Fig. 3. Survival of *Penaeus monodon* after injection with *Vibrio harveyi* isolates. See Table 3 for Groups A to E. In the control group (not shown), none of the shrimp died after injection with artificial sea water (ASW)

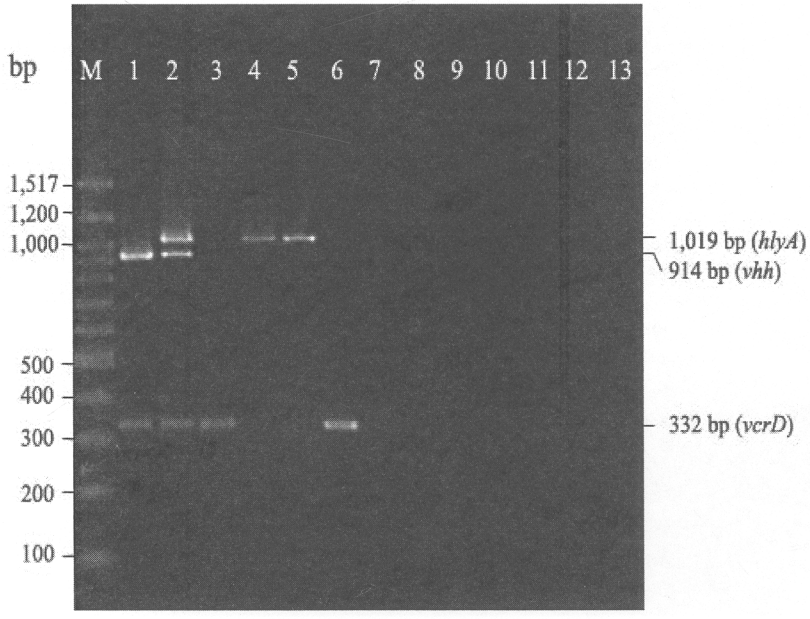

Fig. 4. Specificity of *vcrD*, *vhh*, and *hlyA* primers. Lane M: 100 bp molecular weight marker (New England Biolabs); Lanes 1 to 13, in order: *Vibrio harveyi* NICA, *V. harveyi* HY01, *V. carchariae*, *V. cholerae* O1, *V. cholerae* non-O1, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. campbellii*, *V. furnissii*, *V. fluvialis*, *V. hollisae*, and *Escherichia coli*

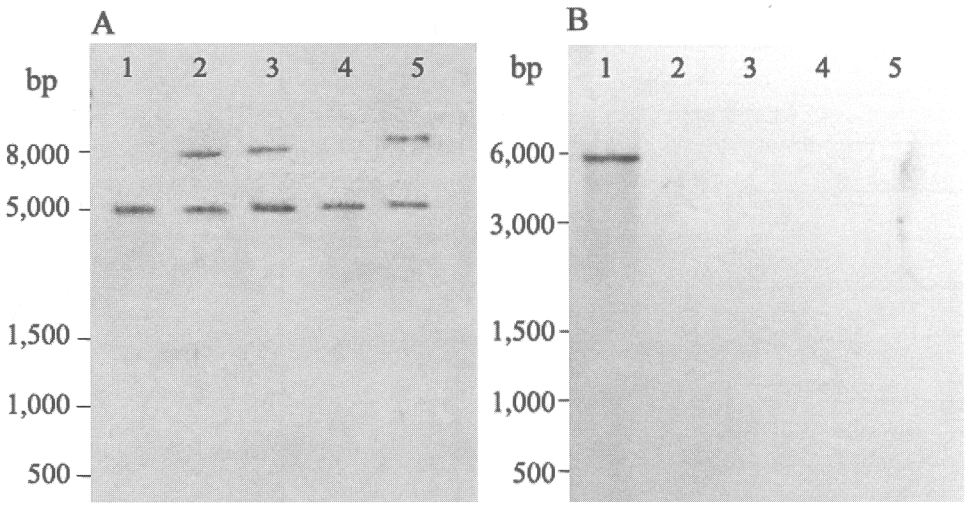

Fig. 5. Southern-blot hybridization analysis to detect (a) *vhh* and (b) *hhl* genes in *Vibrio harveyi*. Genomic DNA was digested with *Eco*RI and hybridized with (a) a 914 bp *vhh*-specific probe and (b) a 1019 bp *hhl*-specific probe under high stringency conditions. Lane 1: *V. harveyi* HY01; Lanes 2 to 5, in order: *V. harveyi* isolates 1, 8, 18, and 24 (molecular weight markers: 1 kb DNA ladder, New England Biolabs)

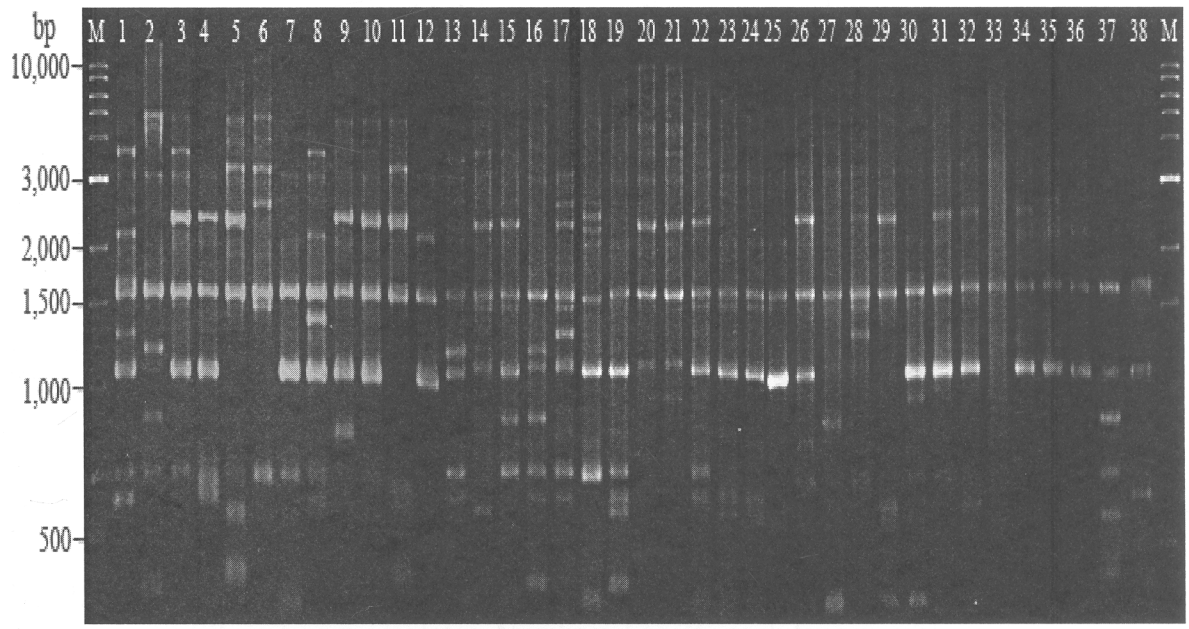

Fig. 6. Random amplified polymorphic DNA (RAPD) profiles of 38 *Vibrio harveyi* isolates. Lanes 1 to 36: each lane number is correlated to the isolate number of *V. harveyi* listed in Table 6; Lanes 37 and 38: *V. harveyi* HY01 and *V. harveyi* BAA-1116, respectively. Molecular weight markers: 1 kb DNA ladder (New England Biolabs)

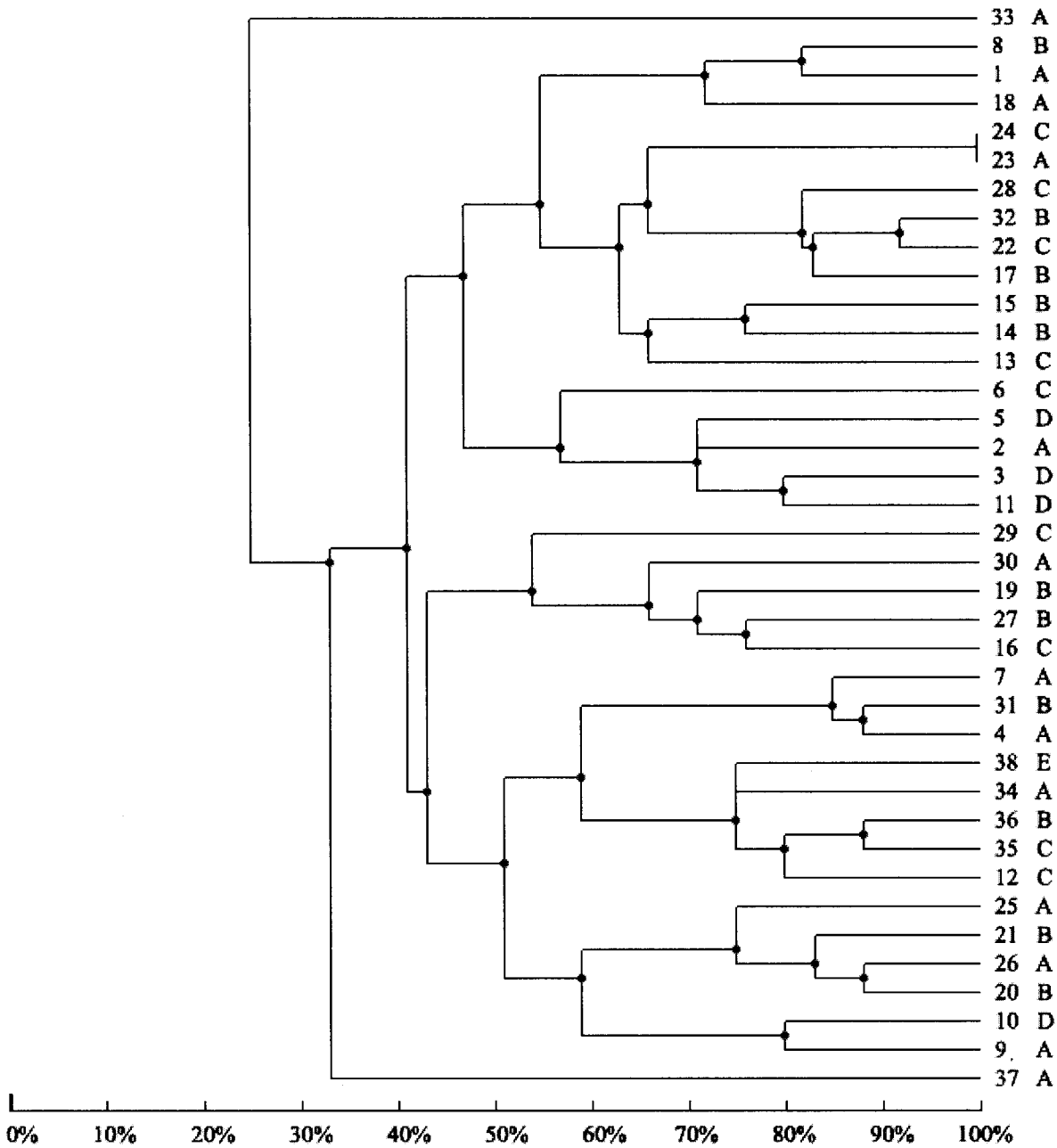

Fig. 7. A dendrogram constructed from random amplified polymorphic DNA (RAPD) profiles of 38 *Vibrio harveyi* isolates. Numbers 1 to 36: each lane number is correlated to the *V. harveyi* isolate number listed in Table 6 and Fig.6. Numbers 37 and 38: *V. harveyi* HY01 and *V. harveyi* BAA-1116, respectively. A, B, C, D, and E indicate the groups of *V. harveyi* listed in Table 6

Discussion

V. harveyi is the most common cause of luminous disease. Some PCR primers have been used to investigate *V. harveyi*; however, they have limitations in either species or strain detection. Primers specific to 16S rDNA did detect *V. harveyi* but a few strains of *V. alginolyticus* also gave positive results (Oakey et al., 2003). Six pairs of primers targetted to different genes of *V. harveyi* were screened and primers designed for *luxN*, a quorum sensing gene were the most specific for *V. harveyi*, however, all tested strains were isolated in Mexico (Hernandez and Olmos, 2004). Conejero and Hedreyda, (2003) demonstrated that some primers were specific for a group of strains of *V. harveyi*. Primers targeted to the *toxR* gene that could produce amplicons from isolates from some countries, did not with isolates from Thailand and Ecuador. Therefore, in this study, specific primers for the PCR detection of *V. harveyi* associated with a luminescence mass death of shrimp in Thailand were developed. Two of the three pairs of primers designed (A1B3 and F1B5) produced an amplicon with *V. hollisae*. Only one pair of primers, A2B3, was specific to *V. harveyi*. It amplified a 363 bp *gyrB* gene fragment of *V. harveyi* NICA isolated from shrimp in Thailand as well as from *V. harveyi* ATCC 14126. No cross reactions were detected with 17 other *Vibrio* species or non-related species *P. damselae*, *A. hydrophila*, *E. coli*, *S. flexneri* and *S. boydii* except *V. carchariae* (Table 2). This was not surprising as amplified fragment length polymorphism (AFLP) of 94 *Vibrio* isolated from marine animals showed *V. harveyi* and *V. carchariae* were in the same cluster and different from *V. alginolyticus*, *V. parahaemolyticus*, *V. campbellii* and *V. natriegens* (Pedersen et al., 1998). Ribotyping and DNA hybridization of *V. harveyi* and *V. carchariae* also showed that they were very similar. In addition, 16S ribosomal DNA sequencing demonstrated that *V. harveyi* and *V. carchariae* formed a single cluster (Gauger and Gomez-Chiarri, 2002). Therefore, it is concluded that *V. carchariae* is the junior synonym of *V. harveyi*. In this study, another two pairs of primers also gave an amplicon with *V. carchariae*. However the A2B3 primers could identify 36 out of 40 isolates from seafood that had been diagnosed as possible *V. harveyi* species by biochemical tests (Table 3). This result indicated the validity of using these primers for identification of *V. harveyi*. Gauger and Gomez-Chiarri (2002), Thompson (2003) and Gomez-Gil et al. (2004) reported that phenotypic characteristics could not clearly distinguish *V. harveyi* from other *Vibrio* species. Thus, we found that four isolates did not give a PCR product.

Some virulence factors of pathogenic strains of *Vibrio harveyi* have not been fully investigated. In the present study, we evaluated the pathogenicity of 38 isolates of *V. harveyi* in a shrimp model and investigated their ability to cause hemolysis on prawn blood agar, including the presence of hemolysin genes and a gene involved in TTSS. Chang et al. (2000) previously demonstrated that prawn blood agar was a better model than sheep blood agar for screening pathogenic bacteria isolated from tiger prawns and for observing correlations between their ability to cause shrimp death and hemolysis. In the present study, 12 of 13 *V. harveyi* isolates (92.3%) in Group A (Table 6) produced hemolysis on prawn blood agar, and this group caused all shrimps to die within 12 h of injection. *V. harveyi* HY01 isolated from a shrimp that had died from luminous vibriosis was also classified in this group. However, 81.8, 66.7, and 50.0% of *V. harveyi* isolates in Groups B, C, and D, respectively, displayed hemolytic activity on prawn blood agar, and these isolates were less

virulent in the shrimp model (Fig. 4). No shrimp deaths were detected after injection with *V. harveyi* BAA-1116 (Group E), and this strain caused no hemolysis and exhibited poor growth on prawn blood agar after 7 d of incubation. It is possible that this strain was sensitive to the Rose Bengal used in the plate medium. Evaluation of the correlation between the hemolytic activity of *V. harveyi* and the deaths of infected shrimps by Pearson's correlation coefficient analysis (r) demonstrated a significant correlation at 24 h after infection ($r = 0.972$, $p < 0.05$). Therefore, the presence of *V. harveyi* hemolysin genes was evaluated. Thirty-four isolates of *V. harveyi*, including *V. harveyi* HY01 and BAA-1116, were shown to be positive for *vhh* by PCR, and the remaining 4 isolates (numbers 1, 18, 8, and 24) were shown to be *vhh*-positive by Southern-blot hybridization (Fig. 5a). Different nucleotide sequences in this gene that did not correspond to the target sequences of the *vhh* PCR primers may be the reason why these 4 isolates produced a negative result in the PCR assay. The Southern-blot hybridization results indicated that there were 1 or 2 copies of *vhh* in these 4 isolates, which correlates with the report of Zhang et al.(2001), who demonstrated duplicate *vhh* genes, designated as *vhhA* and *vhhB*, in *V. harveyi* isolates. However, most isolates examined contained only a single gene, and *vhhA* and *vhhB* were shown to have 98.8% homology (Zhang et al.2001).

It is of interest that *vhh* was detected in all *Vibrio harveyi* isolates but some isolates (isolate numbers 9, 15, 14, 12, 13, 24, 10, and 11) did not show hemolysis on prawn blood agar. This indicates that in some isolates there may be unknown factors that control gene expression and hemolysin production. Zhang & Austin (2000) demonstrated that different strains of *V. harveyi* produce different amounts of hemolysin against erythrocytes of salmon, trout, sheep, rabbit, donkey, and horse. Although hemolysin production against salmon erythrocytes from some isolates had a titer of 1:8 to 1:16 and caused 20 to 40% mortality in rainbow trout, 1 *V. harveyi* isolate with the highest titer (1:256) caused 60% mortality in rainbow trout. Future work to investigate factors that can affect *vhh* gene expression may clarify the correlation between the amount of hemolysin produced and the pathogenicity of *V. harveyi*.

We also found that *Vibrio harveyi* HY01 contains *hhl*, which has a sequence similar to the *hlyA* gene of *V. cholerae*. Therefore, specific primers were designed to detect this gene in other *V. harveyi* isolates. *hhl* was detected in only a few strains of *V. harveyi* and the positive isolates were not classified into Group A, the most pathogenic group (Table 6). Southern-blot hybridization results confirmed that the isolates shown to be *hhl*-negative by PCR did not possess this gene (Fig. 5b). These results indicate that *hhl* is not conserved in *V. harveyi* and might not be a *V. harveyi* virulence determinant. Several hemolysin genes similar to *hlyA* have been reported in *Vibrio* spp.; for example, most clinical and environmental isolates of *V. mimicus* possess *vmh* (Shinoda et al. 2004), and *vvh* and *vahl* have been detected in most isolates of *V. vulnificus* and *V. anguillarum*, respectively (Hirono et al. 1996, Aono et al. 1997). Nucleotide sequence analysis of HY01 *hhl* and *hlyA* genes of *V. cholerae* N16961 (GenBank accession no. AE003853) and *V. cholerae* N86 (GenBank accession no. X51746), *vmh* genes of *V. mimicus* ATCC33653 (GenBank accession no. U68271), *vahl* genes of *V. anguillarum* PT84057 (GenBank accession no. S83534), and *vvh* genes of *V. vulnificus* EDL174 (GenBank accession no. M34670) revealed 86, 86.8, 73, 68.9, and 41% identity, respectively. Therefore, it is possible that the *hhl* detected in a few isolates of *V. harveyi* in the present study might be derived from *V. cholerae* as a discrete genetic unit by horizontal gene transfer. Further

analysis of any mobile genetic elements closely associated with *hhl* may clarify how this gene has appeared in some *V. harveyi* isolates.

Several extracellular products (ECPs), such as a cysteine protease, have been reported in *Vibrio harveyi* (Liu & Lee 1999). Secretion of these ECPs may be controlled through a TTSS that has been detected in many pathogenic bacteria such as *Yersinia* spp., *Shigell*a spp., *Salmonella* spp., *Pseudomonas* spp., and some species of *Vibrio* (Galan et al. 1999, Makino et al. 2003, Dziejman et al. 2005). Park et al. (2004) demonstrated that the TTSSs detected in *V. parahaemolyticus* are involved in cytotoxicity and enterotoxicity. In the present study, *vcrD*, a gene involved in the TTSS of *V. harveyi,* was examined. As it was detected in all isolates of *V. harveyi* except 3 isolates of Group A (Table 6), we concluded that *vcrD* might not be correlated with the pathogenicity of *V. harveyi* in shrimp. The presence of this gene may relate to its persistence in the environment. By hybridization analysis using probes of the *V. cholerae* secretion genes (*vcsV2*, *vcsN2*, and *vcsC2*) and the *V. cholerae* secreted protein gene (*vspD*), Rahman et al. (2008) found that TTSS was not detected in most clinical isolates of *V. cholerae* O1 and O139, but was present in 11.9% of environmental isolates of *V. cholerae* non-O1 and non-O139. This indicates that, in some bacteria, TTSSs might have functions in the environment.

RAPD-PCR has been reported to be a useful technique for distinguishing *Vibrio harveyi* isolates (Hernández & Olmos 2004). In the present study, RAPD analysis of the 38 *V. harveyi* isolates in Groups A to E showed a high degree of genetic diversity and no correlation was detected between DNA profiles of pathogenic and non-pathogenic isolates. This may be because this organism is present in many marine environments and therefore has become associated with a range of animals and environmental factors. As a result, to ensure survival in these diverse habitats, isolates have acquired a range of genes, resulting in the diverse RAPD profiles. Four isolates of *V. harveyi* that possess *hhl* produced different RAPD profiles, which indicates that they are of unrelated origins.

In conclusion, we evaluated the mortality of shrimps infected with *Vibrio harveyi* isolates obtained from fish and shellfish, including 1 isolate (HY01) obtained from a dead shrimp and another (BAA-1116) obtained from sea water, and demonstrated correlations between the isolates that showed hemolytic activity on prawn blood agar and the ability to cause shrimp death. Although *vhh* was detected in all isolates of *V. harveyi*, some isolates showed no hemolytic activity. This indicates that some as-yet-unknown factors might be involved in regulating gene expression. A *V. cholerae hlyA*-like gene, not previously reported in *V. harveyi*, was detected in 4 *V. harveyi* isolates including *V. harveyi* HY01. However, the presence of this gene, including a TTSS gene, *vcrD*, was not related to the death of infected shrimps.

References

Abraham, T.J. and Palaniappan, R. 2004. Distribution of luminous bacteria in semi-intensive penaeid shrimp hatcheries of Tamil Nadu, India. Aquaculture 232: 81-90.

Alsina, M. and Blanch, A.R. 1994. A set of keys for biochemical identification of environmental *Vibrio* spp. J. Appl. Bacteriol. 76: 79–85.

Aono E, Sugita H, Kawasaki J, Sakakibara H, Takahashi T, Endo K, Deguchi Y 1997. Evaluation of the polymerase chain reaction method for identification of *Vibrio vulnificus* isolated from marine environments. J Food Prot 60: 81–83

Bassler BL, Greenberg EP, Stervens AM 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi.* J Bacteriol 179:4043–4045

Baticados, M.C.L., Lavilla-Pitogo, C.R., Cruz-Lacierda, E.R., de la Pena, L.D. and Sunaz, N.A. 1991. Study on the chemical control of luminous bacteria *V. harveyi* and *V. splendidus* isolated from diseased *P. monodon* larvae and rearing water. Dis. Aquat. Organ. 9: 133-139.

Bhoopong P, Palittapongarnpim P, Pomwised R, Kiatkittipong A and others 2007. Variability of the properties of *Vibrio parahaemolyticus* strains isolated from single patients. J Clin Microbiol 45:1544–1550

Cámara M, Hardman A, Williams P, Milton D 2002. Quorum sensing in *Vibrio cholerae.* Nat Genet 32:217–218 Chang CI, Liu WY, Shyu CZ (2000) Use of prawn blood agar hemolysis to screen for bacteria pathogenic to cultured tiger prawns *Penaeus monodon.* Dis Aquat Org 43: 153–157

Chen, S.N., Huang, S.L. and Kou, G.H. 1992. Studies on the epizootiology and pathogenicity of bacterial infections in cultured giant tiger prawns, *Penaeus monodon*, in Taiwan. In: W. Fulks and K.L. Main (Eds). Diseases of Cultured Penaeid Shrimp in Asia and the United States. The Oceanic Institute, Honolulu, pp. 195-205.

Conejero, M.J. and Hedreyda, C.T. 2003. Isolation of partial *toxR* gene of *Vibrio harveyi* and design of *toxR*-targeted PCR primers for species detection. J. Appl. Microbiol. 95: 602-611.

De Clerck, E., Vanhoutte, T., Hebb, T., Geerinck, J., Devos, J. and De Vos, P. 2004. Isolation, characterization, and identification of bacterial contaminants in semifinal gelatin extracts. Appl. Environ. Microbiol. 70 : 3664-3672.

Donabedian H 2003. Quorum sensing and its relevance to infectious diseases. J Infect 46:207–214 Dziejman M, Serruto D, Tam VC, Sturtevant D and others (2005) Genomic characterization of non-O1, non-O139 *Vibrio cholerae* reveals genes for a type III secretion system. Proc Natl Acad Sci USA 102:3465–3470

Dunlap, P.V. and Jennifer, C.A. 2005. Genomic and phylogenetic characterization of luminous bacteria symbiotic with the deep-sea fish *Chlorophthalmus albatrossis* (Aulopiformes: Chlorophthalmidae). Appl. Environ. Microbiol. 71 930-939.

Food and Drug Administration 2001. Bacteriological Analytical Manual. www.cfsan.fda.gov

Galan JE, Collmer A 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science 284:1322–1328

Gauger, E.J. and Gomez-Chiarri, M. 2002. 16S ribosomal DNA sequencing confirms the synonymy of *Vibrio harveyi* and *V. carchariae*. Dis. Aquat. Organ. 52: 39-46.

Gomez-Gil, B., Soto-Rodriguez, S., Garcia-Garca, A., Roque, A., Vazquez-Juarez, R., Thompson, F.L. and Swings, J. 2004. Molecular identification of *Vibrio harveyi*-related isolates associated with diseased aquatic organisms. Microbiology 150: 1769-1777.

Harris, L., Owens, L. and Smith, S. 1996. A selective and differential medium for *V. harveyi*. Appl. Environ. Microbiol. 62, 3548-3550.

Henke JM, Bassler BL 2004a Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. J Bacteriol 186:3794–3805

Henke JM, Bassler BL 2004b Bacterial social engagements. Trends Cell Biol 14:648–656

Hernández G, Olmos J 2004. Molecular identification of pathogenic and nonpathogenic strains of *Vibrio harveyi* using PCR and RAPD. Appl Microbiol Biotechnol 63: 722–727

Hirono I, Masuda T, Aoki T 1996. Cloning and detection of the hemolysin gene of *Vibrio anguillarum*. Microb Pathog 21:173–182

Honda T, Ni Y, Miwatani T 1988. Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. Infect Immun 56:961–965

Hueck CJ 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol Mol Biol Rev 62:379–433

Hsu, H.-C., Lo, C.-F., Lin, S.-C., Liu, K.-F., Peng, S.-E., Chang,Y.-S., Chen, L.-L., Liu, W.-J. and Kou, G.-H. 1999. Studies on effective PCR screening strategies for white spot syndrome virus (WSSV) detection in *Penaeus monodon* brooders. Dis. Aquat. Organ. 39: 13-19.

Karunasagar, I., Pai, R., Malathi, G.R. and Karunasagar, I. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture 128: 203-209.

Karunasagar, I., Otta, S.K. and Karunasagar, I. 1996. Biofilm formation by *Vibrio harveyi* on surfaces. Aquaculture 140: 241–245.

Kashef N, Behzadian-Nejad Q, Najar-Peerayeh S, Mousavi- Hosseini K, Moazzeni M, Djavid GE 2006. Synthesis and characterization of *Pseudomonas aeruginosa* alginate– tetanus toxoid conjugate. J Med Microbiol 55:1441–1446

Kita-Tsukamoto, K., Oyaizu, H., Nanba K. and Simidu, U. 1993. Phylogenetic relationships of marine bacteria, mainly members of the family Vibrionaceae, determined on the basis of 16S rRNA sequences. Int. J. Syst. Bacteriol. 43: 8-19.

Lavilla-Pitogo CR, Baticados MCL, Cruz-Lacierda ER, de la Pena EL 1990. Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. Aquaculture 91:1–13

Liu PC, Lee KK 1999. Cysteine protease is a major exotoxin of pathogenic luminous *Vibrio harveyi* in the tiger prawn, *Penaeus monodon.* Lett Appl Microbiol 28:428–430

Liu, P.-C., Lee, K.-K. and Chen, S.-N. 1996a. Pathogenicity of different isolates of *Vibrio harveyi* in tiger prawn, *Penaeus monodon.* Lett. Appl. Microbiol. 22: 413–416.

Liu, P.-C., Lee, K.-K., Yii, K.-C., Kou, G.-H. and Chen, S.-N. 1996b. Isolation of *Vibrio harveyi* from diseased Kuruma prawns *Penaeus japonicus.* Curr. Microbiol. 33: 129–132.

Makino K, Oshima K, Kurokawa K, Yokoyama K and others 2003. Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae.* Lancet 361:743–749

McMacken, R., Silver, L. and Geogopoulos, C. 1987. DNA replication, In: Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M. and Umbarger, H.E. (Eds), *Escherichia coli* and *Salmonella* Typhimurium: Cellular and Molecular Biology. American Society for Microbiology, Washington, DC, pp. 578-580.

Miller MB, Bassler BL (2001) Quorum sensing in bacteria. Annu Rev Microbiol 55:165–199

Miyamoto Y, Kato T, Obara Y, Akiyama S, Takizawa K, Yamai S 1969. In vitro hemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. J Bacteriol 100:1147–1149

Neter J, Kutner MH, Nachtsheim CJ, Wasserman W 1996. Applied linear statistical models, 4th edn. McGraw-Hill,Chicago, IL

Oakey, H.J., Levy, N., Bourne, D.G. Cullen, B. and Thomas, A. 2003. The use of PCR to aid in the rapid identification of *Vibrio harveyi* isolates. J Appl. Microbiol. 95, 1293-1303.

Park KS, Ono T, Rokuda M, Jang MH, Okuda K, Iida T, Honda T 2004. Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus.* Infect Immun 72:6659–6665

Pedersen, K., Verdonck, L., Austin, B., Austin, D.A., Blanch, A.R., Grimont, P.A.D., Jofre, J., Koblavi, S., Larsen, J.L., Tiainen, T., Vigneulle, M. and Swings, J. 1998. Taxonomic evidence that *Vibrio carchariae* Grims et al. 1985 is a junior synonym of *Vibrio harveyi* (Johnson and Shunk 1936) Baumann et al. 1981. Int. J. Syst. Bacteriol. 48, 749-758.

Prayitno, S.B. and Latchford, J.W. 1995. Experimental infections of crustaceans with luminous bacteria related to *Photobacterium* and *Vibrio*. Effect of salinity and pH on infectiosity. Aquaculture 132: 105–112.

Radu S, Mutalib SA, Rusul G, Ahmad Z and others 1998. Detection of *Escherichia coli* O157:H7 in the beef marketed in Malaysia. Appl Environ Microbiol 64:1153–1156

Rahman MH, Biswas K, Hossain MA, Sack RB, Mekalanos JJ, Faruque SM 2008. Distribution of genes for virulence and ecological fitness among diverse *Vibrio cholerae* population in a cholera endemic area: tracking the evolution of pathogenic strains. DNA Cell Biol 27:347–355

Raungpan, L., Tabkaew, R. and Sangrungruang, K. 1995. Bacterial flora of ponds with different stocking densities of black tiger shrimp, *Penaeus monodon*. In: Shariff, M., Arthur, J.R. and Subasinghe, R.P. (Eds), Diseases in Asian Aquaculture II Fish Health Section, Asian Fisheries Society, Manila, pp. 141-149.

Reed LJ, Muench H 1938. A simple method of estimating fifty percent end point. Am J Hyg 27:493–497

Ruangsri, J., Wannades, M., Wanlem, S., Songnui, A., Arurat, S., Tanmark, N., Patcharat, J. and Supamattaya, K. 2004. Pathogenesis and virulence of *Vibrio harveyi* from southern part of Thailand in black tiger shrimp, *Penaeus monodon* Fabricius. Songklanakarin J. Sci. Technol. 26: 43-54.

Saeed, M.O. 1995. Association of *Vibrio harveyi* with mortalities in cultured marine fish in Kuwait. Aquaculture 136: 21-29.

Sae-Oui, D., Tansutapanit, A. and Ruangpan, L. 1987. *Vibrio harveyi* a causative agent of white shrimp nauplii, *Penaeus merguiensis*.Thai Fish. 2: 177-182.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Shinoda S, Nakagawa T, Shi L, Bi K and others 2004. Distribution of virulence associated genes in *Vibrio mimicus* isolates from clinical and environmental origins. Microbiol Immunol 48:547–551

Soto-Rodriguez SA, Roque A, Lizarraga-Partida ML, Guerra- Flores AL, Gomez-Gil B 2003. Virulence of luminous vibrios to *Artemia franciscana* nauplii. Dis Aquat Org 53: 231–240

Stevens AM, Greenberg EP 1997. Quorum sensing in *Vibrio fisheri*: essential elements for activation of the luminescence genes. J Bacteriol 179:557–562

Song, Y.-L. and Lee, S.P. 1993. Characterization of ecological implication of luminous *Vibrio harveyi* isolated from tiger shrimp (*Penaeus monodon*). Bull. Inst. Zool. Acad. Sin. 32: 217–220,

Sunaryanto, A. and Marium, A. 1986. Occurrence of pathogenic bacteria causing luminescence in panaeid larvae in Indonesian hatcheries. Bull. Brackishwater Aquacul. Dev. Cent. 8: 64-70.

Sung, H.H. and Song, Y.L. 1996. Tissue location of Vibrio antigen delivered by immersion to tiger shrimp (Penaeus monodon). Aquaculture 145, 41-54.

Thaithongnum S, Ratanama P, Weeradechapol K, Sukhoom A, Vuddhakul V 2006. Detection of *V. harveyi* in shrimp postlarvae and hatchery tank water by the Most Probable Number technique with PCR. Aquaculture 261:1–9

Thompson, F.L. 2003. Improved taxonomy of the family Vibrionaceae. Ph.D. thesis, Ghent University.

Vandenberghe, J., Verdonck, L., Robles-Arozarena, R., Rivera, G., Bolland, A., Balladares, M., Gomez-Gil, B., Calderon, J., Sorgeloos, P. and Swings, J. 1999. Vibrios associated with *Litopenaeus vannamei* larvae, postlarvae, broodstock, and hatchery probionts. Appl. Environ. Microbiol. 65: 2592-2597.

Vuddhakul, V., Nakai, T., Matsumoto, C., Oh, T., Nishino, T., Chen, C.H., Nishibuchi, M. and Okuda, J. 2000. Analysis of *gyrB* and *toxR* gene sequences of *Vibrio hollisae* and development of *gyrB*- and *toxR*-targeted PCR methods for isolation of *V. hollisae* from the environment and its identification. Appl. Environ. Microbiol. 66: 3506-3514.

Winzer K, Williams P 2001. Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria. Int J Med Microbiol 291:131–143

Withyachumnarnkul, B. 1999. Results from black tiger shrimp *Penaeus monodon* culture ponds stocked with postlarvae PCR-positive or -negative for white-spot syndrome virus (WSSV). Dis. Aquat. Org. 39: 21-27.

Wootipoom N, Bhoopong P, Pomwised R, Nishibuchi M, Ishibashi M, Vuddhakul V 2007. A decrease in the proportion of infections by pandemic *Vibrio parahaemolyticus* in Hat Yai Hospital, southern Thailand. J Med Microbiol 56:1630–1638

Yamamoto, S. and Harayama, S. 1995. PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. Appl. Environ. Microbiol. 61: 1104-1109.

Yamamoto, S., Kasai, H., Arnold, D.L., Jackson, R.W., Vivian, A. and Harayama, S. 2000. Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. Microbiology 146: 2385-2395.

Yanez, M.A., Catalan, V., Apraiz, D., Figueras, M.J. and Martinez-Murcia, A.J. 2003. Phylogenetic analysis of members of the genus *Aeromonas* based on *gyrB* gene sequences. Int. J. Syst. Evol. Microbiol. 53: 875-883.

Zhang XH, Austin B 2000. Pathogenicity of *Vibrio harveyi* to salmonids. J Fish Dis 23:93–102

Zhang XH, Austin B 2005. Haemolysins in *Vibrio* species. J Appl Microbiol 98:1011–1019

Zhang XH, Meaden PG, Austin B 2001. Duplication of hemolysin genes in a virulent isolate of *Vibrio harveyi.* Appl Environ Microbiol 67:3161–3167