

Shrimp pathogenicity, hemolysis, and the presence of hemolysin and TTSS genes in *Vibrio harveyi* isolated from Thailand

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ABSTRACT: 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 or the pathogenic strains.

KEY WORDS: *Vibrio harveyi* · Type III secretion system · TTSS · *vcrD* · *vhh* · *hhl* · Random amplified polymorphic DNA analysis · RAPD

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INTRODUCTION

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

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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*.

MATERIALS AND METHODS

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 (LD₅₀) 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 × 10⁸ colony-forming units (CFU) ml⁻¹ using a turbidimeter (Oxoid) and 2-fold dilutions were performed to obtain concentrations of bacteria between 4.0 × 10⁵ and 6.4 × 10⁶ CFU.

The *Penaeus monodon* juvenile shrimps used in the present study were 10 to 13 g in weight and 4 to 5 inches (10.2 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 LD₅₀ 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 $4 \times LD_{50}$ (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 1. 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 $10 \times$ PCR buffer (Promega), 1.5 µl of template DNA, 1.6 µl of 25 mM MgCl₂, 5 µl of *vhh*-F and *vhh*-R or *vcrD1* and *vcrD2* primers (2 µM), 0.1 µl of *Taq* poly-

merase (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 *EcoRI* 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 1). 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 $1 \times$ 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

Table 1. 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)
<i>vhh</i> -F	AAGTAATCAGCAGCAGACGAGCG	3006–3919	914
<i>vhh</i> -R	GAGTGGGCAGAAAATCCAGATGG		
<i>vcrD1</i>	TGWRACACGGGTAACGATGA	1333–1664	332
<i>vcrD2</i>	GTAAGCAGATGAGRATCGACGG		
<i>hhl</i> -F	TTCAGAAAGACTTATGGGCTGGG	2287–3305	1019
<i>hhl</i> -R	TAAACACCGTGATAGATTGGGCG		

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).

Table 2. Investigation of the median lethal dose (LD₅₀) of *Vibrio harveyi* strains HY01 and BAA-1116 in *Penaeus monodon*. 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

<i>V. harveyi</i> strain	No. of shrimp dead/total	Cumulative no. shrimp		Mortality (%)
Dose (CFU shrimp ⁻¹)		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

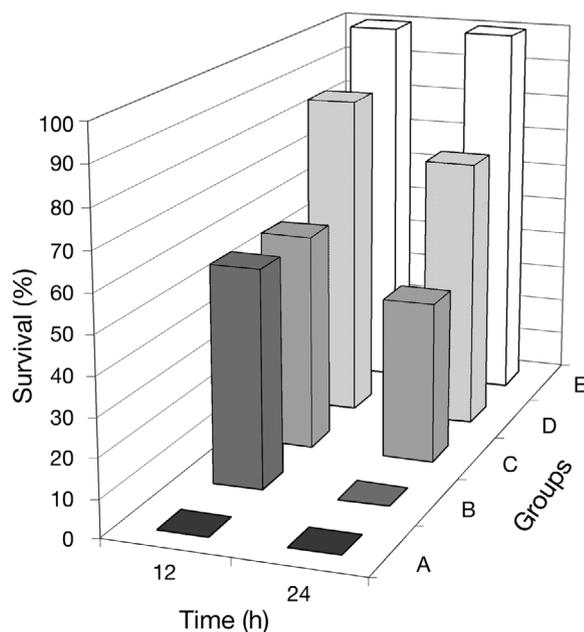


Fig. 1. Survival of *Penaeus monodon* after injection with *Vibrio harveyi* isolates. See Table 3 for Groups A to E. In the control group (not shown), none of the shrimp died after injection with artificial sea water (ASW)

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

RESULTS

Determination of shrimp pathogenicity

The LD₅₀ 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

Table 3. Detection of hemolysis and *vcrD*, *vhh*, and *hhl* genes in *Vibrio harveyi* isolates. +: detected; -: not detected

Isolate no.	Hemolysis on prawn blood agar	PCR detection of			Group
		<i>vcrD</i>	<i>vhh</i>	<i>hhl</i>	
4	+	+	+	-	A
7	+	+	+	-	A
23	+	+	+	-	A
25	+	+	+	-	A
26	+	+	+	-	A
30	+	+	+	-	A
33	+	+	+	-	A
34	+	+	+	-	A
9	-	+	+	-	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	- ^a	+	+	-	E

^aNot detected due to poor growth compared with other strains tested on the same prawn blood agar plates after 7 d of incubation

injection of *V. harveyi* HY01 at concentrations of 6.4×10^6 and 3.2×10^6 CFU shrimp⁻¹, respectively (Table 2). No shrimp deaths were detected after injection of *V. harveyi* BAA-1116. Therefore, the LD₅₀ of *V. harveyi* HY01 was calculated to be 1.9×10^6 CFU shrimp⁻¹. Inoculum concentrations equivalent to $4 \times \text{LD}_{50}$ 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. 1, Table 3), 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% 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% 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 3). 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 1) 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. 2). For the *vcrD* primers, only *V. harveyi*, *V. carchariae*, and *V. parahaemolyticus* gave a positive result with a single 332 bp DNA product.

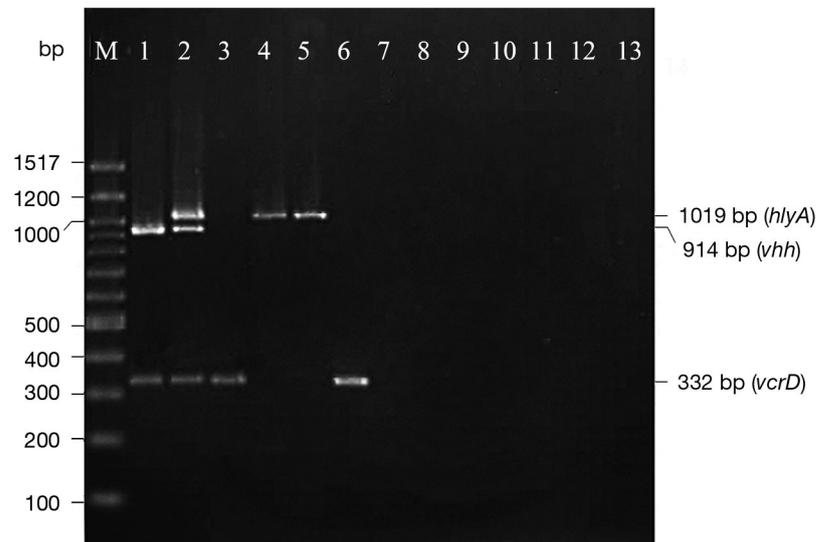


Fig. 2. Specificity of *vcrD*, *vhh*, and *hlyA* primers. Lane M: 100 bp molecular weight marker (New England Biolabs); Lanes 1 to 13, in order: *Vibrio harveyi* NICA, *V. harveyi* HY01, *V. carchariae*, *V. cholerae* O1, *V. cholerae* non-O1, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. campbellii*, *V. furnissii*, *V. fluvialis*, *V. hollisae*, and *Escherichia coli*

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 3). *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 3). 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 3). Therefore, Southern-blot hybridization was performed using the *vhh* and *hhl* probes (Fig. 3), and these isolates (plus isolate number 24) were shown to be *vhh*-positive (Fig. 3a).

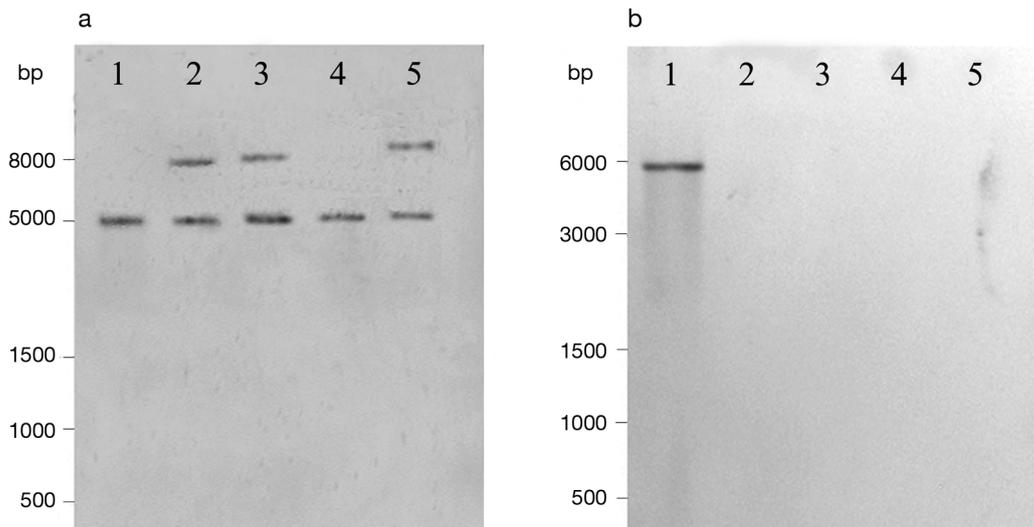


Fig. 3. Southern-blot hybridization analysis to detect (a) *vhh* and (b) *hhl* genes in *Vibrio harveyi*. Genomic DNA was digested with *Eco*RI and hybridized with (a) a 914 bp *vhh*-specific probe and (b) a 1019 bp *hhl*-specific probe under high stringency conditions. Lane 1: *V. harveyi* HY01; Lanes 2 to 5, in order: *V. harveyi* isolates 1, 8, 18, and 24 (molecular weight markers: 1 kb DNA ladder, New England Biolabs)

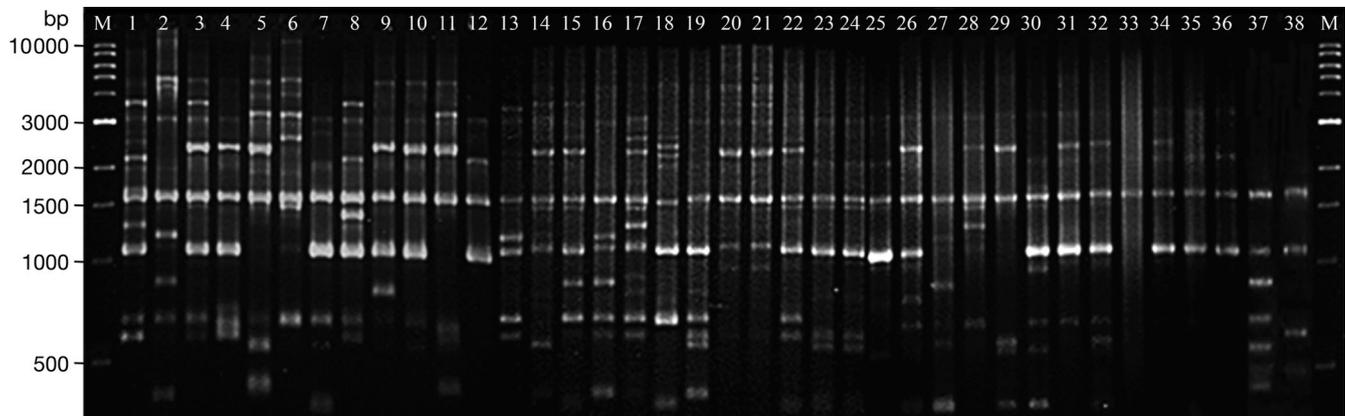


Fig. 4. Random amplified polymorphic DNA (RAPD) profiles of 38 *Vibrio harveyi* isolates. Lanes 1 to 36: each lane number is correlated to the isolate number of *V. harveyi* listed in Table 3; Lanes 37 and 38: *V. harveyi* HY01 and *V. harveyi* BAA-1116, respectively. Molecular weight markers (M): 1 kb DNA ladder (New England Biolabs)

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 12 000 bp. Although all of the isolates shared one 1600 bp amplicon, 16 distinctive DNA profiles at 70% similarity were observed (Figs. 4 & 5). *V. harveyi* HY01 and all 3 *V. harveyi* isolates with *hhl* showed different DNA profiles.

DISCUSSION

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

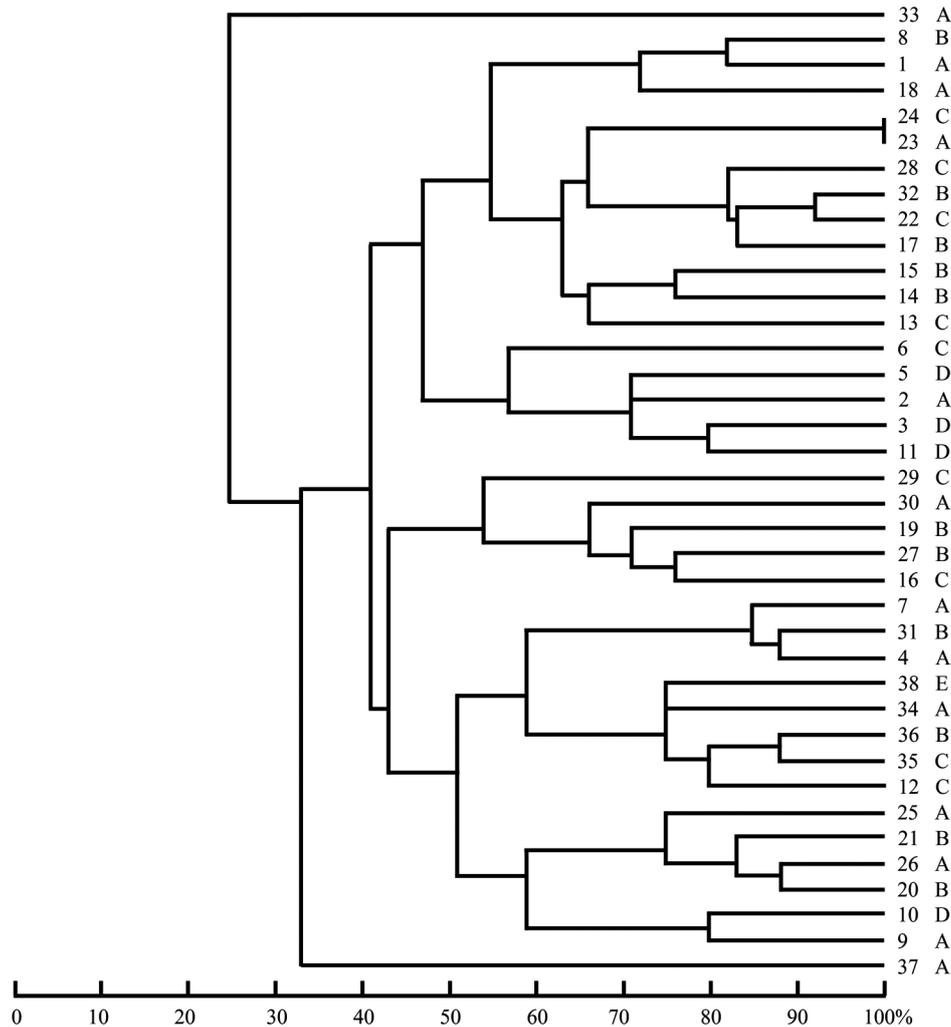


Fig. 5. A dendrogram constructed from random amplified polymorphic DNA (RAPD) profiles of 38 *Vibrio harveyi* isolates. Numbers 1 to 36: each lane number is correlated to the *V. harveyi* isolate number given in Table 3 and Fig. 4. Numbers 37 and 38: *V. harveyi* HY01 and *V. harveyi* BAA-1116, respectively. A, B, C, D, and E indicate the groups of *V. harveyi* listed in Table 3

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 3) 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. 1). 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. 3a). 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 3). Southern-blot hybridization results confirmed that the isolates shown to be *hhl*-negative by PCR did not possess this gene (Fig. 3b). 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., *Shigella* spp., *Salmonella* spp., *Pseudomonas* spp., and some species of *Vibrio* (Galan & Collmer 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 3), 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.

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