Malaysian Journal of Microbiology, Vol 8(2) 2012, pp. 104-115

Characterization and experimental infection of *Vibrio harveyi* isolated from diseased Asian seabass (*Lates calcarifer*)

Julian Ransangan[1*], Tamrin Mohammad Lal[1] and Ahmed H. Al-Harbi[2]

[1]Microbiology and Fish Disease Laboratory, Borneo Marine Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia
[2]Natural Resources and Environment Research Institute, King Abdulaziz City for Science and Technology, P. O. Box 6086 Riyadh, 11442 Saudi Arabia
E.mail: liandra@ums.edu.my

Received 19 September 2011; Received in revised form 31 January 2012; Accepted 23 February 2012

ABSTRACT

Aims: *Vibrio harveyi* causes vibriosis to Asian seabass (*Lates calcarifer*). The disease spreads rapidly among fish stocked in the same cage. It causes high mortality especially in weak and small sized fish stocked at high density in poorly managed net cage. Study to determine the virulence levels of the bacterial pathogen in various aquaculture animals is a key to prevent vibriosis in marine aquaculture.
Methodology and Result: Isolation of bacteria from diseased Asian seabass was done using tryptic soy agar (TSA) and thiosulphate citrate bile sucrose agar (TCBS) plates. Virulence of two strains of *Vibrio harveyi* (VHJR4 and VHJR7) was tested against clinically healthy aquaculture animals. The analysis revealed that the two bacterial strains differ in pathogenicity. The *V. harveyi* strain VHJR7 was virulent to Asian seabass at 1.40 x 10^4 c.f.u. g^{-1}, humpback grouper (*Cromileptis altivelis*) at LD_{50} 8.33 x 10^3 c.f.u. g^{-1} and black tiger shrimp (*Penaeus monodon*) at LD_{50} 3.26 x 10^4 c.f.u. g^{-1} , respectively. The *V. harveyi* strain VHJR4 was not virulent to Asian seabass and humpback grouper but it caused mortality to black tiger shrimp at LD_{50} 1.32 x 10^6 c.f.u. g^{-1}. Phenotypically, the two strains shared most of the biochemical features except that the *V. harveyi* strain VHJR7 was a urease positive and grew at 8.5 % NaCl, and at 10 °C. The percentage similarity of nucleotide sequences of 16S rDNA in *V. harveyi* VHJR4 and *V. harveyi* VHJR7 was higher (99 %) but reduced at 95 % in hemolysin gene.
Conclusion, significance and impact of study: Pathogenic strain of *V. harveyi* causes mortality and affects aquaculture production of Asian seabass. Hence, vaccine development against the bacterial pathogen is urgently needed for sustainability of Asian seabass aquaculture in Malaysia.

Keywords: *Vibrio harveyi*, virulence, Asian seabass, vibriosis

INTRODUCTION

Vibrio harveyi is a Gram-negative bacterium, ubiquitous in the marine environments and is found free-living in the water column, and in the gut of some marine animals. The bacterium is a causative agent of vibriosis that capable of infecting wide range of aquatic animals including penaeids (Liu *et al.,* 1996; Abraham *et al.,* 1997; Alvarez *et al.,* 1998; Robertson *et al.,* 1998), sea horse (Tendencia, 2004), bivalves (Pass *et al.,* 1987), cephalopods (Ramesh *et al.,* 1999), marine teleosts (Yii *et al.,* 1997; Soffientino *et al.,* 1999; Zhang & Austin 2000; Thompson *et al.,* 2002; Tendencia, 2002) and elasmobranches (Grimes *et al.,* 1984). *V. harveyi* caused chronic skin ulcer in shark (Bertone *et al.,* 1996), gastro-enteritis in cultured groupers (Yii *et al.,* 1997; Lee *et al.,* 2002), red drum (Liu *et al.,* 2003), and cultured cobia (Liu *et al.,* 2004). The bacterium was also reported to cause deep dermal lesions in wild specimens of jack crevalle (Kraxberger-Beatty *et al.,* 1990). In addition, it caused mortalities in farmed sole (Zorrilla *et al.,* 2003), cultured brown spotted grouper and silvery black porgy (Saeed, 1995), and cage cultured grouper (Qin *et al.,* 2006). It caused eye lesions in milkfish (Ishimaru & Muroga, 1997), common snook (Kraxberger-Beatty *et al.,* 1990), and sunfish (Hispano *et al.,* 1997). In cultured Asian seabass (*Lates calcarifer*), however, the bacterium causes vibriosis that is characterized by anorexia, darkening of the whole fish, local hemorrhagic ulcers on the mouth or skin surface, tail and fin rot, focal necrotic lesions in the muscle and swollen intestine, and eye opacity (Tendencia, 2002; Ransangan & Mustafa, 2009). The virulence of *V. harveyi* is reported dependent on host species (Vera *et al.,* 1992), doses, time exposure and age of host species (Jun & Huai-shu, 1998), and pathogenic factors of the bacterial strains (Gomez-Gill *et al.,* 1998).

This paper describes the virulence of two strains of *V. harveyi* isolated from diseased Asian seabass cultured in the open net cages installed in coastal waters of Sabah, Malaysia to Asian sea bass (*Lates calcarifer*), *humpback* grouper (*Cromileptis altivelis*) and black tiger shrimp

*Corresponding author

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 1: *Vibrio* strains used in the present study

Strain	Origin	Isolated organ	Locality/year	Reference
V. carchariae (*V. harveyi*) ATCC 35084	*Carcharhinus plumbeus*	Kidney	USA/1982	Grimes *et al.* (1984); Pedersen *et al.* (1998)
V. harveyi VHJR4	*Lates calcarifer*	Kidney	Malaysia/2008	This study
V. harveyi VHJR7	*Lates calcarifer*	Heart	Malaysia/2008	This study

(*Penaeus monodon*).

MATERIALS AND METHODS

Bacterial characterization

Two strains of *V. harveyi* designated as VHJR4 and VHJR7 isolated from diseased Asian seabass, *Lates calcarifer* (Bloch) cultured in open net cages in Sabah, Malaysia during the vibriosis outbreak in February 2008 (Ransangan & Mustafa, 2009) were used in this study. These strains were isolated from head kidney and heart of diseased seabass (weighing 5-7 g, water temperature 28 °C) using tryptic soy agar (Difco, Detroit, MI; supplemented with 2 % NaCl; TSA) and/or thiosulphate citrate bile salt sucrose agar (TCBS; Difco) plates. The pure cultures were stored in tryptic soy broth (Difco; +2 % NaCl; 25 % glycerol) at -86 °C. Subsequently, the bacteria were characterized using the standard biochemical method as described by Alsina & Blanch (1994) and identified using 16S rRNA and hemolysin gene sequencing. For the temperature tolerance test, each bacterial isolate was incubated at 5, 10, 12, 15, 20, 25, 30, 35, 37 and 40 °C. Tolerance to NaCl was determined for 0, 0.5, 3, 4, 5, 6, 8, 8.5, 9.0 and 10 % (w/v) NaCl in tryptic soy broth. Hemolytic activity of the bacterial strains was tested against tilapia and cow bloods. All the tests were performed in triplicate at 28 °C and incubated for 24 h. The reactions were compared with a reference strain of *V. carchariae* (*V. harveyi*) ATCC 35084 (Grimes *et al.,* 1984). The origin of each bacterial strain used in this study is shown in Table 1.

Isolation of genomic DNA

Genomic DNA from all bacterial strains was extracted using the proteinase K extraction method. Briefly, the bacteria were inoculated in sterile 5ml TSB (Difco, Becton Dickinson, Maryland, USA) and incubated at 28 °C overnight. Bacterial cells were collected from 1.0 ml of the bacterial suspension by centrifugation at 9,000 rpm for 10 min at 4 °C. The bacterial cells were incubated with lysis buffer (600 µl 1x TE buffer, 30 µl of 10 % SDS (w/v) and 3 µl of 20 mg/ml proteinase K (Sigma)) at 37 °C for 1 h after which the mixture was added with 100µl of CTAB/NaCl of solution and further incubated at 65 °C for 10 min. Subsequently, the mixture was treated with equal volume of chloroform:isoamylalcohol (24:1), and precipitated by centrifugation at 9,000 rpm for 10 min at 4 °C. Then, 600 µl of the aqueous phase was carefully transferred to a fresh microfuge tube with equal volumes of phenol: chloroform: isoamylalcohol (25:24:1) and centrifuged at 8,000 rpm for 10 min at 4 °C. Thereafter, DNA pellet was precipitated from 500 µl of aqueous phase using equal volume of chilled isopropanol and centrifuged at 13,000 rpm for 15 min at 4 °C. Finally, the DNA pellet was washed with 1.0 ml of chilled 70 % ethanol and dried briefly before dissolving into 50 µl 1X TE buffer. The concentration of the extracted DNA was determined using GeneQuant™ RNA/DNA calculator.

PCR amplification, cloning and sequencing

The total genomic DNA extracted from all bacterial strains was amplified for 16S ribosomal RNA and hemolysin genes. The partial sequence of 16S rDNA was amplified using PCR primers designed based on the partial 16S rDNA sequence (GQ370528) of *Vibrio harveyi* ATCC 35084. The forward (3F: CCTGAAGTGGGGGATAACC) and reverse (2R: ACGTCGTCCCCACCTTCCTC) primers were designed from nucleotides 2-20 and 1061-1042, respectively. The hemolysin gene was amplified using PCR primers (VHF2: GAGGACGTTTGGTGAGATAA and VHR2: ACGACGAATACAATCTCTGG) described by Zhang *et al.* (2001). The PCR amplifications for both genes were conducted in 25 µl total reaction (12.5 µl of PCR Master Mix (Promega, Madison, Wisconsin), 1.0 µl of each 10 µM forward and reverse primers, 1.0 µl DNA template (0.307 µg/µl) and 9.5 µl nuclease-free water). The PCR amplification was carried out one cycle at 95 °C for 3 min, 30 cycles at 95 °C for 1 min, 58 °C for 1 min and at 72 °C for 1 min, and finally one cycle at 72 °C for 5 min. PCR products were purified using *AccuPrep*™ PCR purification Kit (Bioneer Corporation, Seoul, Korea) according to the manufacturer's instruction. Two microlitre (2.0 µl) of the PCR product was cloned using pGEM®-T Easy (Promega) cloning vector as prescribed by the manufacturer's manual. The plasmid was purified using PureLink™ Quick Plasmid Miniprep Kit (Invitogen) following the manufacturer's instructions. Finally, 20 µl of the purified plasmid harbouring DNA inserts as determined by *EcoR1* digestion analysis was sequenced using M13 forward and reverse primers (Macrogen, DNA sequencing service, Seoul, Korea). The nucleotide sequences of the 16S rDNA and hemolysin gene were further analyzed by using DNASTAR (Windows version 5.05, Wisconsin, USA) for the construction of phylogenetic tree and the calculation of percentage of identity.

Antibiotic Sensitivity Assay

Stock cultures of the bacterial strains (VHJR4 and VHJR7) were grown on TSA for 24 h at 28 °C. The bacteria were

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

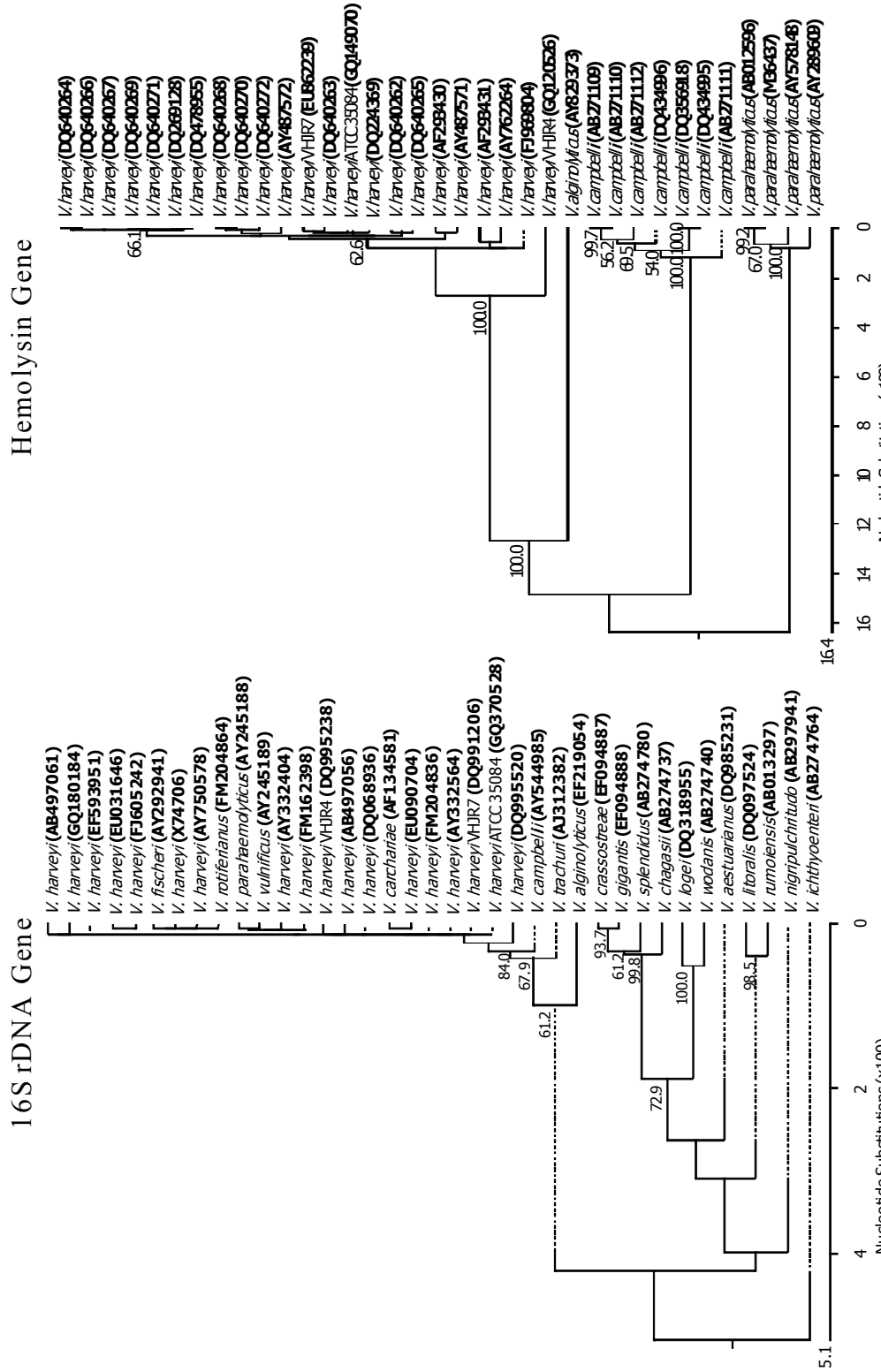

Figure 1: Phylogenetic tree based on 37 nucleotide sequences (890bp) and 35 nucleotide sequences (1257bp) of 16S ribosomal DNA (left) and hemolysin (right) genes, respectively, of *Vibrio* species. This tree was constructed by using the Clustal W method in the MegAlign package, DNASTAR Ver. 5.05, Wisconsin, USA.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 2: Phenotypic traits of *V. harveyi* strains VHJR4 and VHJR7 in comparison with reference strain of *Vibrio harveyi* (ATCC 35084)[a].

Character	ATCC 35084	VHJR4	VHJR7
Gram stain	-	-	-
Motility	-	-	-
Oxidase	+	+	+
Catalase	+	+	+
Voges-Proskauer Test	-	-	-
Indole production	+	+	+
Citrate utilization	-	-	-
O/F glucose	F	F	F
Gas from glucose	-	-	-
Growth at 5 °C	-	-	-
Growth at 10 °C	+	-	+
Growth at 12 °C	+	+	+
Growth at 15 °C	+	+	+
Growth at 20 °C	+	+	+
Growth at 25 °C	+	+	+
Growth at 28 °C	+	+	+
Growth at 30 °C	+	+	+
Growth at 35 °C	+	+	+
Growth at 37 °C	+	+	+
Growth at 40 °C	-	-	-
Growth at 0 % NaCl	-	-	-
Growth at 0.5 % NaCl	+	+	+
Growth at 3 % NaCl	+	+	+
Growth at 5 % NaCl	+	+	+
Growth at 6 % NaCl	+	+	+
Growth at 8 % NaCl	+	+	+
Growth at 8.5 % NaCl	+	-	+
Growth at 9.0 % NaCl	+	-	-
Growth at 10 % NaCl	-	-	-
Arginine dihydrolase	-	-	-
Lysine decarboxylase	+	+	+
Phenylalanine agar	-	-	-
β-galactosidase (ONPG)	-	-	-
Methyl-red test	+	+	+
Urease	+	-	+
Acid from D-fructose	+	+	+
Acid from D-cellobiose	+	+	+
Acid from D-mannose	+	+	+
Acid from D-sorbitol	-	-	-
Acid from L-arabinose	-	-	-
Acid from D-dextrose	+	+	+
Acid from D-sucrose	+	+	+
Acid from D-maltose	+	+	+
Acid from D-mannitol	+	+	+
Acid from D-lactose	-	-	-
Acid from D-salicin	-	-	-
Acid from D-raffinose	-	-	-
Acid from D-galactose	+	+	+
Acid from L-rhamnose	-	-	-
β-Hemolytic on cow's blood	+	+	+
β-Hemolytic on tilapia's blood	+	+	+
Gelatin Agar	+	+	+
Bioluminescence	-	-	-
Growth on TCBS agar	Y	Y	Y

[a] Note: +, positive; -, negative; Y, yellow; F, fermentative.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

suspended in sterile phosphate buffered saline (PBS: 0.8775 % NaCl, 0.02 % KCl, 0.02 % KH_2PO_4, 0.115 % Na_2HPO_4, 10 % glycerol, pH 7.2) and diluted to a turbidity equivalent to a MacFarland No. 0.5 standard solution (0.5 ml $BaSO_4$ + 99.5 ml 0.36 N HCl). The bacterial suspension (0.1 ml) was spread onto Mueller-Hinton agar (Difco) and antibiotic discs then added as described by Koneman *et al.* (1988). The antibiotics discs (Oxoid) used in the assay included ampicilin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), furazolidone (100µg), kanamycin (30 µg), nalidixic acid (3 µg), neomycin (10µg), nitrofurantoin (300 µg), novobiocin (5 µg), oxolinic acid (2 µg), oxytetracycline (30 µg), penicillin G (10 units), streptomycin (25 µg), sulphamethoxazole (100µg), tetracycline (10 µg) and vancomycin (30 µg). The plates were incubated at 35 °C for 18 h and inhibition zones were measured using a graduated ruler (0.5 mm), and the average result was recorded to the nearest millimeter.

Fish and virulence tests

Asian seabass (*Lates calcarifer*), humpback grouper (*Cromileptis altivelis*) and black tiger shrimp (*Penaeus monodon*) weighing 5-7 g in total body weight were held in tanks (1000L) supplied with aerated 29 ppt seawater at 28-29 °C. The two bacterial strains (VHJR4 and VHJR7) were selected for virulence tests. The lethal dose-50 % end point (LD_{50}) tests, with batches of 20 animals per dose, were conducted by intraperitoneal (i.p) injection in seabass and humback grouper, and intramuscular (i.m) injection at the 5th abdominal segment in black tiger shrimp with 24 h bacterial suspension (10^4-10^8 c.f.u. animal^{-1}) into the test animals (Trevors and Lusty, 1985). Sterile PBS was injected into other groups of animals as parallel controls. The LD_{50} values were calculated using the method of Reed & Muench (1938). Mortalities were recorded daily for 11 days post challenge. Reisolation and identification of the bacteria from kidney of moribund animals after bacterial challenge were conducted by using TSA and TCBS agar plates.

RESULTS

Bacterial characterization

All bacterial strains examined in this study were glucose fermentative, non-motile, oxidase and catalase positive. They recorded negative in Vogues-Proskauer, citrate utilization, arginine dehydrolase, phenylalanine agar, and β-galactosidase tests but positive in lysine decarboxylase, methyl red and indole production tests. Two bacterial strains (VHJR7 and ATCC 35084) were able to utilize urea while VHJR4 was not. All bacterial strains produced acid from carbohydrates fermentation test (D-fructose, D-cellobiose, D-glucose, D-mannose, D-dextrose, D-sucrose, D-maltose, D-mannitol and D-galactose) but no gas produced. However, no acid was produced from other carbohydrates such as D-lactose, D-salicin, D-raffinose, and L-rhamnose (Table 2). All bacterial strains grew well on TCBS agar with yellow colonies. All the organisms grew at 12-37 °C, but not at 5, and 40 °C. However, *V. harveyi* (ATCC 35084) and (VHJR7) grew at 10 °C. All bacterial strains grew well in 0.5-8 % (w/v) sodium chloride but not in 0 % and 10 %, respectively. The *V. harveyi* ATCC 35084 grew at 8.5 % and 9.0 % (w/v) sodium chloride whereas *V. harveyi* VHJR7 grew only at 8.5 % but not at 9 %. All bacteria were able to utilize 10 % gelatin and produced β-hemolytic activity on both tilapia and cow blood agars.

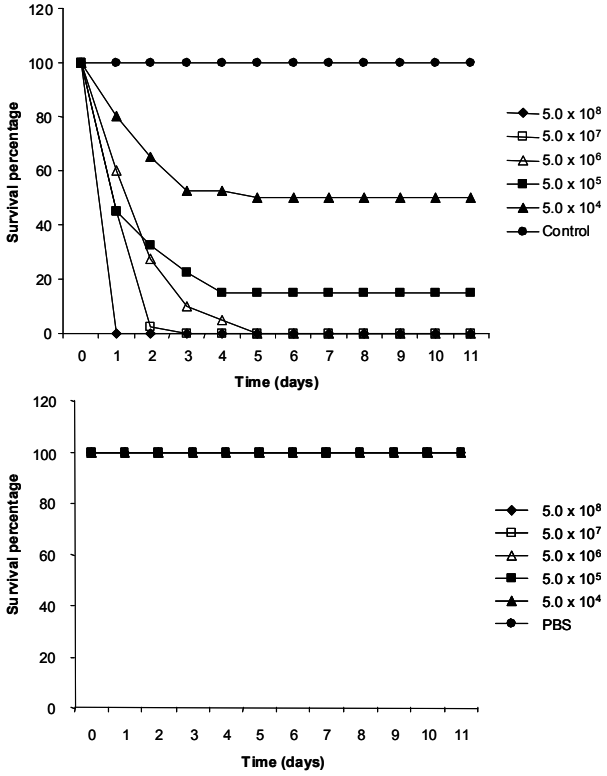

Figure 2: Average survival percentage of Asian seabass (*L. calcarifer*) challenged with *V. harveyi* strain VHJR7 (upper) and VHJR4 (lower). ◆ fish challenged with 5.0 x 10^8 c.f.u. fish^{-1}; □ fish challenged with 5.0 x 10^7 c.f.u. fish^{-1}; △ fish challenged with 5.0 x 10^6 c.f.u. fish^{-1}; ■ fish challenged with 5.0 x 10^5 c.f.u. fish^{-1}; ▲ fish challenged with 5.0 x 10^4 c.f.u. fish^{-1}; ● fish challenged with sterile PBS.

Nucleotide sequencing analysis

The PCR amplifications of 16S rDNA from the two bacterial strains (VHJR4 and VHJR7) were successfully carried out using PCR primers designed in the study. The PCR products (1060bp) of 16S rDNA from the two bacterial strains were successfully cloned into pGEM-T Easy Cloning vector and sequenced. The partial sequences of 16S rDNA were made available in the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 3: Sensitivity of *V. harveyi* VHJR4 and *V. harveyi* VHJR7 to various antibiotics.

Antibiotics	Disc content (µg)	Sensitivity [a]
Ampicilin	10	R
Chloramphenicol	30	S
Ciprofloxacin	5	S
Furazolidone	100	S
Kanamycin	30	MS
Nalidixic acid	3	S
Neomycin	10	R
Nitrofurantoin	300	S
Novobiocin	5	MS
Oxolinic acid	2	S
Oxytetracycline	30	S
Penicilin G	10 units	R
Streptomycin	25	S
Sulphamethoxazole	100	S
Tetracycline	30	MS
Vancomycin	30	R

[a] R: resistance; S: sensitive; MS: moderately sensitive.

sequences of 16S rDNA were made available in the GenBank with accession numbers DQ995238 and DQ991206, respectively. Although the nucleotide sequence analysis of the 16S rDNA fragments (nt100-990nt of *V. harveyi* ATCC 35084 (GQ370528)) from the two bacteria revealed high percentage of identity (>99.9) to 16S rDNA nucleotide sequences belonging to *V. harveyi*, they were also showed high similarity (94-99.8 %) to other *Vibrio* species (Figure 1).

The complete coding sequences (1.3kb) of hemolysin gene from the bacterial strains (VHJR4 and VHJR7) were also successfully cloned and sequenced. The nucleotide sequences were then deposited into GenBank with the accession numbers GQ120526 and EU862239, respectively. Although the nucleotide sequence analysis of hemolysin gene (nt65-nt1321 of *V. harveyi* ATCC 35084 (GQ149070)) in VHJR7 revealed high percentage of identity (>99 %) to *V. harveyi*, the VHJR4 showed a reduced (94.0-95.4 %) identity. However, the nucleotide sequences from both bacterial strains showed lower percentage of similarities when compared to other closely related *Vibrio* species such as *V. alginolyticus* (84.6 %), *V. campbellii* (78.9-79.2 %) and *V. parahaemolyticus* (77.1-77.9 %). On the basis of sequencing results, the two bacterial strains examined in this study were identified as *V. harveyi*.

Antibiotic susceptibility assay

The antibiotic susceptibility assay (Table 3) revealed that the two *V. harveyi* strains (VHJR4 and VHJR7) were sensitive to chloramphenicol, ciprofloxacin, furazolidone, nalidixic acid, nitrofurantoin, oxolinic acid oxytetracycline, streptomycin, sulphamethoxazole and trimethoprim. Both strains showed intermediate reaction to kanamycin, novobiocin and tetracycline. However, they were resistant to ampicilin, neomycin, penicillin and vancomycin.

Virulence assays

When the virulence of the two *V. harveyi* strains (VHJR4 and VHJR7) was evaluated, it was found that *V. harveyi* VHJR7 highly virulent to seabass at LD_{50} $1.40x10^4$ c.f.u. g^{-1} fish (Figure 2), humpback grouper at LD_{50} 8.33×10^3 c.f.u. g^{-1} fish (Figure 3) and to black tiger shrimp at LD_{50} 3.26×10^4 c.f.u. g^{-1} shrimp (Figure 4). Although *V. harveyi* VHJR4 did not produce mortality to seabass and humpback grouper (Figure 2 and 3; Table 4), it caused mortality to shrimp at LD_{50} 1.32×10^6 c.f.u. g^{-1} (Figure 4 and Table 4). Pure cultures of bacteria as homologous colonies were recovered from the kidney of the moribund fish and hepatopancreas of shrimp after bacterial challenge and identified as the same species. No mortality was observed in the controls injected with sterile PBS.

DISCUSSION

The isolation of *V. harveyi* from diseased seabass in the present study is not surprising as the bacterial pathogen has been isolated in a number of diseased marine animals including Asian seabass (Tendencia, 2002), abalone (Nishimori *et al.,* 1998; Nicolas *et al.,* 2002), groupers (Rasheed, 1989; Yii *et al.,* 1997), shrimps (Jiravanichpaisal *et al.,* 1994; Vandenberghe *et al.,* 1998), red drum (Liu *et al.,* 2003) and sharks (Grimes *et al.,* 1984). Although *V. harveyi* caused gastroenteritis in

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

cultured flounder (Soffientino *et al.,* 1999; Gauger *et al.,*2006), grouper (Yii *et al.,* 1997), red drum (Liu *et al.,* 2003), seabream (Liu *et al.,* 1996) and cobia (Liu *et al.,* 2004), it was reported to cause deep skin lesions, hemorrhagic of fin base and anus, tail and fin rot, and eye opacity in the Asian seabass (Ransangan & Mustafa, 2009).

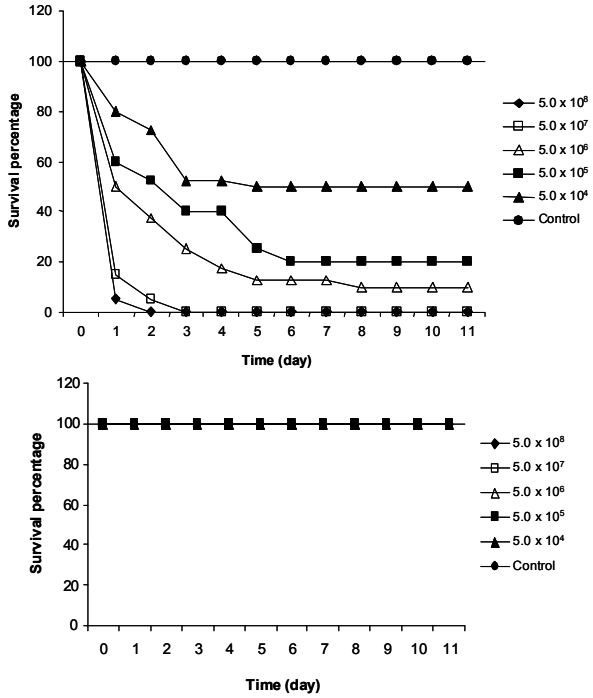

Figure 3: Average survival percentage of humpback grouper (*C. altivelis*) challenged with *V. harveyi* strain VHJR7 (upper) and VHJR4 (lower). ◆ fish challenged with 5.0 x 10^8 c.f.u. $fish^{-1}$; □ fish challenged with 5.0 x 10^7 c.f.u. $fish^{-1}$; △ fish challenged with 5.0 x 10^6 c.f.u. $fish^{-1}$; ■ fish challenged with 5.0 x 10^5 c.f.u. $fish^{-1}$; ▲ fish challenged with 5.0 x 10^4 c.f.u. $fish^{-1}$; ● fish challenged with sterile PBS.

Because *V. harveyi* has been known to be phenotypically heterogenous (Grimes *et al.,* 1993; Alsina and Blanch, 1994; Vandenberghe *et al.,* 2003) and reported to contain mobile genetic elements such as bacteriophages (Oakey & Owens, 2000) that often contribute new phenotypic characteristics (Munro *et al.,* 2003), the biochemical and/or physiological tests cannot precisely identify the pathogen (Vandenberghe *et al.,* 2003). This appears to be the case in the present study.

Although 16S rDNA sequencing has been proven useful for classification and identification of bacterial species (Kolbert & Persing, 1999), the finding of this study shows that high percentage of similarity (>94 %) of nucleotide sequences of the 16S rDNA in both strains of *V. harveyi* (VHJR4 and VHJR7) compared to their closely related species may not be sufficient to correctly identify the two bacteria into species level. In the past, similar findings were also reported by several authors (Gomez-Gil *et al.,* 2004; Chiang *et al.,* 2006).

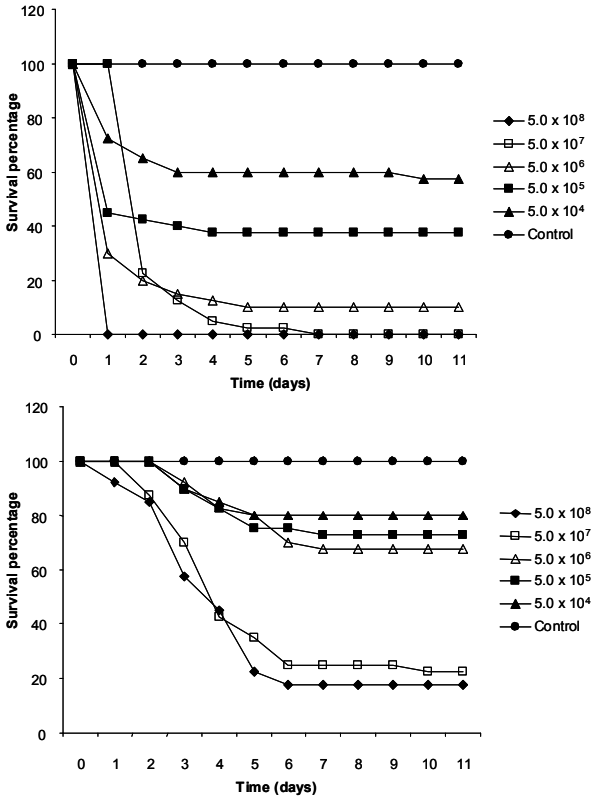

Figure 4: Average survival percentage of black tiger shrimp (*P. monodon*) challenged with *V. harveyi* strain VHJR7 (upper) and VHJR4 (lower). ◆ fish challenged with 5.0 x 10^8 c.f.u. $shrimp^{-1}$; □ fish challenged with 5.0 x 10^7 c.f.u. $shrimp^{-1}$; △ fish challenged with 5.0 x 10^6 c.f.u. $shrimp^{-1}$; ■ fish challenged with 5.0 x 10^5 c.f.u. $shrimp^{-1}$; ▲ fish challenged with 5.0 x 10^4 c.f.u. $shrimp^{-1}$; ● fish challenged with sterile PBS.

Sequencing of hemolysin gene has been used in the identification of closely related *Vibrio* species (Conejero & Hedreyda, 2004). In the present study, the result from sequencing of hemolysin gene has clearly shown that the two bacterial strains examined are highly identical to *V. harveyi* than to other closely related species such as *V. alginolyticus*, *V. campbellii* and *V. parahaemolyticus*.

V. harveyi strain VHJR7 was shown to be highly virulent to seabass, with average LD_{50} of 1.40 x 10^4 c.f.u. g^{-1} fish. It is also virulent to humpback grouper and black tiger shrimp with average LD_{50} of 8.33 x 10^3 c.f.u. g^{-1} fish and association of *V. harveyi* in diseased marine animals with 3.26 x 10^4 c.f.u. g^{-1} shrimp, respectively. This is not a surprising finding as many studies have reported the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 4: LD_{50} values of the two *V. harveyi* strains against Asian seabass (*Lates calcarifer*), humpback grouper (*C. altivelis*) and black tiger shrimp (*Penaeus monodon*).

Fish Samples	Bacterial Samples	Dose (c.f.u. g^{-1} body weight)	Mortality (%)	LD_{50} Value (c.f.u. g^{-1} body weight)
	PBS (Control)		0	
Asian Seabass	*V. harveyi* VHJR7	5.0×10^8	100	1.40×10^4
		5.0×10^7	99.4	
		5.0×10^6	95.8	
		5.0×10^5	84.4	
		5.0×10^4	40.0	
	V. harveyi VHJR4 [b]	5.0×10^8	0	-
		5.0×10^7	0	
		5.0×10^6	0	
		5.0×10^5	0	
		5.0×10^4	0	
Humpback Grouper	*V. harveyi* VHJR7	5.0×10^8	100	8.33×10^3
		5.0×10^7	98.4	
		5.0×10^6	93.6	
		5.0×10^5	78.8	
		5.0×10^4	50.0	
	V. harveyi VHJR4	5.0×10^8	0	-
		5.0×10^7	0	
		5.0×10^6	0	
		5.0×10^5	0	
		5.0×10^4	0	
Black Tiger Shrimp	*V. harveyi* VHJR7	5.0×10^8	100	3.26×10^4
		5.0×10^7	98.5	
		5.0×10^6	93.0	
		5.0×10^5	66.9	
		5.0×10^4	25.5	
	V. harveyi VHJR4	5.0×10^8	92.6	1.32×10^6
		5.0×10^7	78.7	
		5.0×10^6	42.8	
		5.0×10^5	21.8	
		5.0×10^4	12.7	

different virulence levels. Previous challenge studies involving *V. harveyi* found that the pathogen produced LD_{50} values higher than 2×10^7 cfu g^{-1} in grouper (Yii *et al.,* 1997) and in red drum (Liu *et al.,* 2003). In the study by Saeed (1995), he has reported that *V. harveyi* produced 50% mortality to silvery black porgy (*Acanthopagrus cuvieri*) and brown-spotted grouper (*Epinephelus tauvina*) at $4.90 \pm 0.2 \times 10^7$ c.f.u $fish^{-1}$ and $1.56 \pm 0.19 \times 10^9$c.f.u $fish^{-1}$, respectively. However, virulence study by Liu *et al.* (2004) revealed that *V. harveyi* strain c3d1 was highly virulent to cobia at LD_{50} 7.48×10^4 cfu g^{-1} which is in agreement with the present finding. *V. harveyi* isolated from diseased marine fishes were also reported virulent to the olive flounder (*Paralichthys olivaceus*) and black rockfish (*Sebastes schlegeli*) with LD_{50} of 2.48×10^5-8.76×10^7 and 2.0×10^4-2.52×10^6 cfu g^{-1} fish, respectively (Won and Park, 2008). Also, Zhang and Austin (2000) found that *V. harveyi* (VIB 654, VIB 571 and VIB 572) were virulent to rainbow trout and Atlantic salmon with LD_{50} ranged from 2.51×10^5 to 5.32×10^5 cells/fish. In contrast, the *V. harveyi* strain VHJR4 in this study was not virulent to seabass and humpback grouper but it was slightly virulent to black tiger shrimp with LD_{50} 1.32×10^6 cell/shrimp. The difference in the virulence levels of *V. harveyi* reported in this study with previous studies revealed that pathogenicity of *V. harveyi* is dependent on species (Vera *et al.,* 1992), doses used, time of exposure and age of challenged animals (Jun & Huai-shu, 1998), and the pathogenic factors of the strains employed (Gomez-Gill *et al.,* 1998).

Some studies indicated that virulence factors in *V. harveyi* can be contributed from toxins that either having protease or hemolysin activities (Fukasawa *et al.,* 1989; Liu *et al.,* 1997; Svitil *et al.,* 1997; Zhang & Austin 2000; Zhang *et al.,* 2001; Liu and Lee., 1999). Other studies, however, have shown that the pathogenicity of *V. harveyi* was derived from phage in which genes coding for toxin production were acquired by gene transduction (Morris & Robert, 1995). In addition, a phage infecting *V. harveyi* named VHML (*Vibrio harveyi* Mio-virus like) has been isolated by several authors (Ruangpan *et al.,* 1999; Oakey

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

& Owens, 2000; Oakey *et al.,* 2002) which may strengthen the role of phage in virulence of this group of bacterial pathogen. It was also found that experimental infection of VHML enhanced the toxicity of *V. harveyi* (Austin *et al.,* 2003; Munro *et al.,* 2003). The toxin expression in bacteria may be controlled by gene transduction but some bacteria have been found to express toxin by a process called quorum sensing (Surette & Basler, 1999; Tammy *et al.,* 1999; Bernd *et al.,* 2001; Costi *et al.,* 2002). In this study, the level of pathogenicity in *V. harveyi* VHJR7 was more related to gene transduction in nature rather than quorum sensing since the pathogen is capable of producing mortality in the test animals even in the presence of low colony forming unit. In contrast, the pathogenicity of *V. harveyi* VHJR4 is more related to quorum sensing process whereby more cells of the bacterium are required to produce mortality in shrimp.

The urea-hydrolysing activity may potentially be used as a marker in determining pathogenicity within the *Vibrio* species. In the past, *V. parahaemolyticus* having urea-hydrolysing activity was reported to cause gastroenteritis in human (Kaysner *et al.,* 1994). The occurrence of urea-hydrolysing activity in *V. harveyi* VHJR7 which was virulent to both fish and shrimp in the present study, however, requires further investigation.

Results from the antibiotic susceptibility tests against the two strains of *V. harveyi* examined in this study revealed that they were resistant to ampicilin, penicilin G and vancomycin. However, they were found sensitive to chloramphenicol, ciprofloxacin, nalidixic acid, nitrofurantoin, oxolinic acid and oxytetracycline. Because prolonged usage of antibiotics may cause bacterial pathogens to develop resistance (Miranda & Zemelman, 2002; Sahul Hameed & Balasubramanian, 2000; Karunasagar *et al.,* 1994), their use in the aquaculture may not be advisable. This requires other means of controlling vibriosis in aquaculture to be developed. Although the development of vaccine against *V. harveyi* is still at the preliminary stage, experiments at laboratory level are already showing some interesting results (Zhu *et al.,* 2006; Ningqiu *et al.,* 2008). The fact that the *V. harveyi* VHJR4 is not virulent to fish, it can have the advantages to be developed into live vaccine against vibriosis in marine fish aquaculture in the future.

CONCLUSION

Pathogenic strain of *V. harveyi* causes mortality and affects aquaculture production of Asian seabass. Hence, vaccine development against the bacterial pathogen is urgently needed for the sake of sustainability of Asian seabass aquaculture in Malaysia.

ACKNOWLEDGEMENTS

The authors express special thank to the Department of Fisheries, Sabah for providing experimental animals. This work was funded by the Centre of Research and Innovation, Universiti Malaysia Sabah under the research grant no. FRGS-44.

REFERENCES

Abraham, T.J., Manley, R., Palaniappan, R. and Dhevedaran, K. (1997). Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* isolated from disease penaeid shrimp. Journal of Aquaculture in the Tropics **12: 1 – 8.**

Alsina, M. and Blanch, A.R. (1994). A set of keys for biochemical identification of environmental *Vibrio* species. Journal of Applied Bacteriology **76: 79-85.**

Alvarez, J.D., Austin, B., Alvarez, A.M. and Reyes, H. (1998). *Vibrio harveyi*: a pathogen of penaeid shrimps and fish in Venezuela. Journal of Fish Diseases **21: 313-316.**

Austin, B., Pride, A.C. and Rhodie, G.A. (2003). Association of a bacteriophage with virulence in *Vibrio harveyi*. Journal of Fish Diseases **26: 55-58.**

Bernd, K., Michael, D.P.B., Bettina, A.B., Markus, H. and Andreas, P.(2001). Group A streptococcal growth phase-associated virulence factor regulation by a novel operon (*fas*) with homologies to two-component-type regulators requires a small RNA molecule. Molecular Microbiology **39: 392-406.**

Bertone, S., Gili, C., Moizo, A. and Calegari, L. (1996). *Vibrio carchariae* associated with a chronic skin ulcer on a shark, *Carcharinus plumbeus* (Nardo). Journal of Fish Diseases **19: 429–434.**

Chiang, Y.C., Yang, C.Y., Li, C., Ho, Y.C., Lin, C.K. and Tsen, H.-Y.(2006). Identification of *Bacillus* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp. with 16S ribosomal DNA-based oligonucleotide array hybridization. International Journal of Food Microbiology **107: 131–137**.

Conejero, M.J.U. and Hedreyda, C.T. (2004). PCR detection of hemolysin (*vhh*) gene in *Vibrio harveyi*. Journal of General and Applied Microbiology **50: 137-142**.

Costi, D.S., Eleftherios, M., Kavindra, V.S, Xiang, Q., Danielle, A.G., Barbara, E.M., Frederick, M.A. and Stephen, B.C. (2002). Virulence effect of *Enterococcus faecalis* protease genes and the quorum-sensing locus *fsr* in *Caenorhabditis elegans* and mice. Infection and Immunity **70: 5647-5650**.

Fukasawa, S., Miyahira, M., Hosoda, M. and Kurata, M. (1989). Properties of the proteinases from a luminous bacterium, *Vibrio harveyi* strain FLN-77. Chemical and Pharmaceutical Bulletin **37: 204-205**.

Gauger, E., Smolowitz, R., Uhlinger, K., Casey, J. and Gómez-Chiarri, M. (2006). *Vibrio harveyi* and other bacterial pathogens in cultured summer flounder, *Paralichthys dentatus*. Aquaculture **260: 10-20.**

Gomez-Gil, B., Soto-Rodriguez, S., Garcia-Gasca, 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.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Gomez-Gill, B., Herrera-Vega, M. A., Abreu-Gobois, F. A. and Roque, A. (1998). Bioencapsulation of two diferent *Vibrio* species in nauplii of the brine shrimp (*Artemia franciscana*). Applied and Environmental Microbiology **64: 2318–2322**.

Grimes, D.J., Brayton, P., Colwell, R.R. and Ruber, S.H. (1984). *Vibrio* species associated with mortality of sharks held in captivity. Microbiol Ecology **10: 271-282**.

Grimes, D.J., Jacobs, D., Swartz, D.G., Brayton, P. and Colwell, R.R. (1993). Numerical taxonomy of Gram-negative, oxidase-positive rod from carcharid sharks. International Journal of Systematics and Bacteriology **43: 88 – 89.**

Hispano, C., Nebra, Y. and Blanch, A. (1997). Isolation of *Vibrio harveyi* from an ocular lesion in the short sunfish (*Mola mola*). Bulletin of European Association of Fish Pathologists **17: 104-107.**

Ishimaru, K. and Muroga, K. (1997). Taxonomical re-examination of two pathogenic *Vibrio* species isolated from milkfish and swimming crab. Fish Pathology **32: 59–64.**

Jiravanichpaisal, P., Miyazaki, T. and Limsuwan, C. (1994). Histopathology, biochemistry and pathogenicity of *Vibrio harveyi* infecting black tiger prawn *Penaeus monodon*. Journal of Aquatic Animal Health **6: 27-35.**

Jun L.I. and Huai-Shu, X. (1998). Isolation and biological characteristics of *Vibrio harveyi* affecting hatchery-reared *Penaeus chinensis* larvae. Chinese Journal of Oceanology and Limnology **29: 353–361**.

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

Kaysner, C.A., Abeyta, C., Trost, P.A., Wetherington, J.H., Jinneman, K.C., Hill, W.E. and Wekell, M.M. (1994). Urea hydrolysis can predict the potential pathogencity of *Vibrio parahaemolyticus* strains isolated in the Pacific Northwest. Applied and Environmental Microbiology **60: 3020-3022.**

Kolbert, C.P. and Persing, D.H. (1999). Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. Current Opinion in Microbiology **2: 299-305**

Koneman, E.W., Allen, S.D., Dowell, J.V., Janda, W.M., Sommers, H.M. and Winn, W.C. (1988). Color atlas and textbook of diagnostic microbiology. Philadelphia Press, USA.**pp. 983-993.**

Kraxberger-Beatty, T., McGarey D, Grier, H. and Lim, D. (1990). *Vibrio harveyi*, an opportunistic pathogen of common snook, *Centropomus undecimalis* (Bloch), held in captivity. Journal of Fish Diseases **13: 557-560**.

Lee, K.K., Liu, P.C. and Chuang, W.H. (2002). Pathogenesis of gastroenteritis caused by *Vibrio carchariae* in cultured marine fish. Marine Biotechnology **4: 267-277.**

Lee, K.K., Liu, P.C. and Chen, Y.L. (1999). Electrophoretic characterization of a novel cysteine protease produced by *Vibrio harveyi*. Electrophoresis **20: 3343-3346**.

Liu, P.C. and Lee, K.K. (1999). Cystein protease is a major extoxin of pathogenic luminous *Vibrio harveyi* in the tiger prawn, *Penaeus monodon*. Letters in Applied Microbiology **28: 428-430.**

Liu, P.C., Chuang, W.H. and Lee, K.K. (2003). Infectious gastroenteritis caused by *Vibrio harveyi* (*V. carchariae*) in cultured red drum, *Sciaenops ocellatus*. Journal of Applied Ichthyology **19: 59–61**.

Liu, P.C., Lee, K.K., Tu, C.C. and Chen, S.N. (1997). Purification and characterization of a cysteine protease produced by pathogenic luminous *Vibrio harveyi*. Current Microbiology **35: 32-39.**

Liu, P.C., Lee, K.K., Yii, K.C., Kou, G.H. and Chen, S.N. (1996). Isolation of *Vibrio harveyi* from diseased kuruma prawns *Penaeus japonicus*. Current Microbiology **33: 129-132.**

Liu, P.C., Lin, J.Y., Chuang, W.H. and Lee, K.K. (2004). Isolation and characterization of pathogenic *Vibrio harveyi* (*V. carchariae*) from the farmed marine cobia fish *Rachycentron canadum* L. with gastroenteritis syndrome. World Journal of Microbiology and Biotechnology **20: 495-499**.

Miranda, C.D. and Zemelman, R. (2002). Bacterial resistance to oxytetracycline in Chilean salmon farming. Aquaculture **212: 31-47.**

Morris, P. and Robert, G.H. (1995). Classification of isolates of *Vibrio harveyi* virulent to *Penaeus monodon* larvae by protein profile analysis and M13 DNA fingerprinting. Diseases of Aquatic Organisms **21: 61-68.**

Munro, J., Oakey, H.J., Bromage, E. and Owens, L. (2003). Experimental bacteriophage-mediated virulence in strains of *Vibrio harveyi*. Diseases of Aquatic Organisms **54: 187-194.**

Nicolas, J.L., Basuyaux, O., Mazurie, J. and Thebault, A. (2002). *Vibrio carchariae*, a pathogen of the abalone *Haliotis tuberculata*. Diseases of Aquatic Organisms **50: 35-43.**

Ningqiu, L., Junjie, B., Shuqin, W., Xiaozhe, F., Haihua, L., Xing, Y. and Cunbin, S. (2008). An outer membrane protein, OmpK, is an effective vaccine candidate for *Vibrio harveyi* in Orange-spotted grouper (*Epinephelus coioides*). Fish Shellfish Immunology **25: 829–833.**

Nishimori, E., Hasegawa, O., Numata, T. and Wakabayashi, H. (1998). *Vibrio carchariae* causes mass mortalities in Japanese abalone *Sulculus diversicolor supratexta*. Fish Pathology **33: 495-502.**

Oakey, H.J. and Owens, L. (2000). A new bacteriophage, VHML, isolated from a toxin-producing strain of *Vibrio harveyi* in tropical Australia. Journal of Applied Microbiology **89: 702-709.**

Oakey, H.J., Cullen, B.R. and Owens, L. (2002). The complete nucleotide sequence of the *Vibrio harveyi* bacteriophage VHML. Journal of Applied Microbiology **93: 1089-1098.**

Pass, D., Dybdahl, R. and Mannion, M. (1987). Investigation into the causes of mortality of the pearl

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

oyster, *Pinctada maxima* (Jamson), in Western Australia. Aquaculture **65: 149-169.**

Pedersen, K., Verdonck, L., Austin, D., Blanch A., Grimont, P., Jofre, J., Koblavi, S., Larsen, J., Tiainen, T., Vigneullle, M. and Swings, J. (1998). Taxonomic evidence that *Vibrio carcharidae* Grimes *et al.,* 1985 is a junior synonym of *Vibrio harveyi* (Johnson and Shunk 1936, Baumann *et al.*, 1981). International Journal of Systematic Bacteriology **48: 749-758.**

Qin, Y.X., Wang, J., Su, Y.Q., Wang, D.X. and Chen, X.Z. (2006). Studies on the pathogenic bacterium of ulcer disease in *Epinephelus awoara*. Acta Oceanology Sinica **25: 154–159.**

Ramesh, A., Loganathan, B.G. and Venugopahm, V. K. (1999). Seasonal distribution of luminous bacteria in the sediments of tropical estuary. Journal of General and Applied Microbiology **35: 363-368.**

Ransangan, J. and Mustafa, S. 2009. Identification of *Vibrio harveyi* isolated from diseased Asian seabass (*Lates calcarifer*) by use of 16S ribosomal DNA sequencing. Journal of Aquatic Animal Health **21:150-155.**

Rasheed, V. (1989). Diseases of cultured brown-spotted grouper *Epinephelus tauvina* and silvery black porgy *Acanthopagrus cuviere* in Kuwait. Journal of Aquatic Animal Health **1: 102-107.**

Reed, L.J. and Muench, H. (1938). A simple method of estimating fifty percent end points. American Journal of Hygine **27: 493-497.**

Robertson, P.A., Calderon, J., Carrera, L., Stark, J., Zherdmant, M. and Austin, B. (1998). Experimental *Vibrio harveyi* infections in *Penaeus vannamei* larvae. Diseases of Aquatic Organisms **32: 151-155.**

Ruangpan, L., Danayadol, Y., Direkbusarakom, S., Siurairatana, S. and Flegel, T.W. (1999). Lethal toxicity of *Vibrio harveyi* to cultivated *Penaeus monodon* induced by a bacteriophage. Diseases of Aquatic Organisms **35: 195-201.**

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

Sahul Hameed, A.S. and Balasubramanian, G. (2000). Antibiotic resistance in bacteria isolated from *Artemia nauplii* and efficacy of formaldehyde to control bacterial load. Aquaculture **183: 195-205.**

Soffientino, B., Gwaltney, T., Nelson, D.R., Specker, J.L., Mauel, M. and Gómez-Chiarri, M. (1999). Infectious necrotizing enteritis and mortality caused by *Vibrio carchariae* in summer flounder *Paralichthys dentatus* during intensive culture. Diseases of Aquatic Organisms **38: 201–210.**

Surette, M.G. and Bassler, B.L. (1999). Regulation of autoinducer production in *Salmonella typhimurium*. Molecular Microbiology **31: 585-595.**

Svitil, A.L., Chadhain, S.M. and Moore, J.A. (1997). Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. Applied and Environmental Microbiology **63: 408-413.**

Tammy, M.R., Allison, F.G., Maria, A.S., Richard, G.B., Mark, S.S. and Barry, K.H. (1999). Characterization of the *SarA* virulence gene regulator of *Staphylococcus aureus*. Molecular Microbiology **33: 307-316.**

Tendencia, E.A. (2002). *Vibrio harveyi* isolated from cage-cultured seabass *Lates calcarifer* Bloch in the Philippines. Aquaculture Research **33: 455-458.**

Tendencia, E.A. (2004). The first report of *Vibrio harveyi* infection in the sea horse *Hippocampus kuda* Bleekers 1852 in the Philippines. Aquaculture Research **3: 1292–1294.**

Thompson, F.L., Hoste, B., Vandemeulebroecke, K., Engelbeen, K., Denys, R. and Swings., J. (2002). *Vibrio trachuri* Iwamoto *et al.* 1995 is a junior synonym of *Vibrio harveyi* (Johnson and Shunk 1936) Baumann *et al.* 1981. International Journal of Systematics and Evolutionary Microbiology **52: 973-976.**

Trevors, J.T. and Lasty, C.W. (1985). A basic microcomputer program for calculating LD_{50} values. Water, Air and Soil Pollution **24: 432-442.**

Vandenberghe, J. Thompson, F.L., Gomez-Gil, B. and Swings, J. (2003). Phenotypic diversity amongst *Vibrio* isolates from marine aquaculture systems. Aquaculture **219: 9-20**.

Vandenberghe, J., Li, Y., Verdonck, L., Li, J., Sorgeloos, P., Xu, H.S. and Swings, J. (1998). *Vibrios* associated with *Penaeus chinensis* larvae in Chinese shrimp hatcheries. Aquaculture **169: 121-132.**

Vaseeharan, B., Ramasamy, P., Murugan, T. and Chen, J.C. (2005). In vitro susceptibility of antibiotics against *Vibrio* spp. and *Aeromonas* spp. isolated from *Penaeus monodon* hatcheries and ponds. International Journal of Antimicrobiol Agents **26: 285-291.**

Vera, P., Navas, J.I. and Quintero, M.C. (1992). Experimental study of the virulence of three species of *Vibrio* bacteria in *Penaeus japonicus* (Bate 1881) juveniles. Aquaculture **107: 119-123.**

Waldor, M.K. and Mekalanos, J.J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. Science **28: 1910-1914.**

Won, K. M. and Park, S. I. (2008). Pathogenicity of *Vibrio harveyi* to cultured marine fishes in Korea. Aquaculture **285: 8-13.**

Yii, K.C., Yang, T.I. and Lee, K.K. (1997). Isolation and characterization of *Vibrio carcharieae*, a causative agent of gastroenteritis in the groupers, *Epinephelus coioides*. Current Microbiology **35: 109-115.**

Zhang, X.H. and Austin, B. (2000). Pathogenicity of *Vibrio harveyi* to salmonids. Journal of Fish Diseases **23: 93-102.**

Zhang, H.H., Meaden, P. G. and Austin, B. (2001). Duplication of hemolysin genes in a virulent isolate of *Vibrio harveyi*. Applied and Environmental Microbiology **67: 3161 – 3167.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Zhu, K., Chi, Z., Li, J., Zhang, F., Li, M., Yasoda, H.N. and Wu, L. (2006). The surface of haemolysin from *Vibrio harveyi* on yeast cells and their potential applications as live vaccine in marine fish. **Vaccine 24: 6046 – 6052.**

Zorrilla, I., Chabrillon, M., Arijo, S., Diaz-Rosales, P., Martinez-Manzanares, E., Balebona, M.C. and Morinigo, M.A. (2003). Bacteria recovered from diseased cultured gilthead sea bream (*Sparus aurata* L.) in southwestern Spain. Aquaculture **218: 11-20.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538