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Phylogenetic Analysis of Atypical Hemolysin Gene in *Vibrio campbellii* and Effects of Cultivation Salinity and pH on Hemolytic Activity and Virulence

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Abstract

The purposes of this study were to analyze the atypical hemolysin gene of *V. campbellii* isolates, and to evaluate the effects of cultivation salinity (0.5, 1.5, and 3.0 % NaCl) and pH (5.0, 7.3, and 8.6) on the hemolytic activity and virulence of *V. campbellii*. Phylogenetic analysis of atypical hemolysin gene sequences obtained from *V. campbellii* demonstrated 84 - 85 % identity with the *hlyA* of *V. cholerae*. *V. campbellii* grown at 1.5 or 3.0 % NaCl, which exhibited significant higher hemolytic activity compared to those previously grown at 0.5 % NaCl. Maximum hemolytic activity was observed among acid-adapted *V. campbellii* which was previously grown at pH 5.0 for 5 h. Likewise, its virulence against *Galleria mellonella* was enhanced (~20 times) in comparison to that of non-adapted *V. campbellii*. Based on our results, it seems that *V. campbellii* might have acquired hemolysin gene from *V. cholerae*. Moreover, both cultivation salinity and pH are deemed important for the hemolytic activity and virulence of *V. campbellii*. This will be useful for the environmental control of this pathogen in aquaculture.

Keywords: Hemolytic activity, *hlyA*, *Galleria mellonella*, *Vibrio campbellii*, virulence

Introduction

The outbreak of shrimp disease, especially from *Vibrio* belonging to the Harveyi clade (e.g., *Vibrio harveyi, V. campbellii,* and *V. parahaemolyticus*), has been the primary factor which limits the production growth of farmed shrimp [1]. A marine bacterium *V. campbellii* is widely distributed in aquaculture environments, including rearing water, near-shore seawater, sediment, and animal samples [2,3]. *V. campbellii* is closely related to *V. harveyi* and shares more than 97 % similarity in 16S rRNA sequence [4]. Previously identified *V. harveyi* isolates associated with diseased aquatic organisms were in fact *V. campbellii* [4-8]. Many studies indicate that *V. campbellii* is an emerging aquaculture pathogen associated with shrimp disease in aquaculture [9-11].

Recent studies have demonstrated that hemolysins of vibrios play an important role in virulence, in both animals and humans [12-16]. Furthermore, it has been suggested to be the main virulence factor of *V. campbellii* [9]. *V. campbellii* hemolysin is encoded by *vch* gene which is typical for the Harveyi clade vibrios (*vhh* of *V. harveyi* and *tlh* of *V. parahaemolyticus*), and is present in all *V. campbellii* isolates. However, some *V. campbellii* isolates harbor *V. cholerae hlyA*-like hemolysin gene [5,17,18].

Some strains of *V. campbellii* are highly pathogenic. Environmental factors, especially salinity and pH, are significantly involved in virulence of many species of vibrios. For example, salinity affects *V. harveyi* virulence against *Fenneropenaeus indicus*, and additionally plays an important role in bacterial attachment and virulence of *V. alginolyticus* and *V. anguillarum* in a fish model [19,20]. Low pH regulates hemolysin production, in which virulence has been reported in *V. vulinificus* [21].*V. campbellii*

frequently encounters salinity and pH stress in marine environments. However, knowledge on how environmental factors affect *V. campbellii* virulence is still limited.

Hence, in the present study, phylogenetic analysis of the atypical *V. campbellii* hemolysin gene was performed to demonstrate its similarity among *hlyA* homologues. The effects of cultivation salinity and pH on hemolytic activity and virulence of *V. campbellii* toward *Galleria mellonella* larvae were also carried out to obtain a clearer understanding of the factors influencing *V. campbellii* virulence. An understanding of these factors may help to develop proper shrimp pond management to minimize the serious impact of *V. campbellii* infection in shrimp.

Materials and methods

Bacterial strains

A total of four *V. campbellii* (previously identified as *V. harveyi*) strains that were used in this study were previously isolated from shrimp samples (**Table 1**). Species was confirmed by Polymerase Chain Reaction (PCR) [22]. All strains had been tested for the presence of the hemolysin genes and had been investigated for their pathogenicity to shrimp [5]. The strains were grown in trypticase soy agar (TSA) or trypticase soy broth (TSB) containing 1.5 % (w/v) NaCl (TSB-1.5) and were incubated for 18 - 20 h at 30 °C. In some experiments, the bacteria were grown in TSB containing 0.5 % NaCl (TSB-0.5) or 3.0 % NaCl (TSB-3). Glycerol (15 %) stocks of the strain were kept at −80 °C for long-term storage.

For non-*campbellii* species listed in **Table 1**, only the sequences of *hlyA* gene homologues obtained from GenBank database were used.

Analysis of atypical hemolysin gene of *V. campbellii*

Chromosomal DNA was extracted through a boiling method [23]. *V. cholerae hlyA*-like hemolysin gene in *V. campbellii* was amplified by PCR using primers *hhl*-F and *hhl*-R, as previously reported [5]. The PCR products were sequenced (Macrogen, Seoul, Korea) and the resulting gene sequences were analyzed using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Partial nucleotide sequences of the hemolysin gene of *V. campbellii* obtained in this study were aligned with the sequences of *hlyA* homologues in various *Vibrio* spp. and *Aeromonas hydrophila* which were also obtained from GenBank database (http://www.ncbi.nlm.nih.gov). A phylogenetic tree was generated using the neighbor-joining method with the Molecular Evolutionary Genetics Analysis (MEGA) program version 6.0, based on alignments from CLUSTAL W [24]. Bootstrap values were obtained from 1000 replicates.

Nucleotide sequence accession numbers

The nucleotide sequence data were deposited in GenBank under the accession numbers MF319496 to MF319499.

Growth of bacteria under various cultivation conditions

The isolated *V. campbellii* HY01 from the shrimp that had died from luminous vibriosis [23] was used as a representative strain for this study. It was incubated under various conditions. Three salinity conditions (0.5, 1.5, and 3.0 % NaCl) and three pH conditions (5.0, 7.3, and 8.6) were assessed. All possible combinations (n = 9) of salinity and pH were tested. All tests were conducted in triplicates and bacterial growth was monitored every 60 min by measuring absorbance at 600 nm using a spectrophotometer (BioTex, Vermont, USA). Bacterial growth at each salinity/pH combination described above was compared [25]. The growth rates (μ) were calculated using the following equation:

$$N_t = N_0 \times e^{(\mu t)} \quad (1)$$

where N_0 and N_t are the cellular concentrations at the start of the experiment and time t (in hours), respectively.

Effect of salinity and pH on hemolytic activity

To study the role of the culture conditions on hemolytic activity, *V. campbellii* HY01 was initially cultured for 5 h under the various conditions described above. Cell-associated hemolytic activity was quantified as the percentage of red blood cells lysed in liquid suspension using a 96-well microtiter plate assay [25]. In brief, the bacterial cells and defibrinated sheep erythrocytes were washed twice with phosphate buffered saline (PBS). The bacterial cell suspensions were adjusted to a final concentration of 1.5×10^8 colony forming units (CFU)/ml, and 190 µl of each suspension was thoroughly mixed with 10 µl of packed sheep erythrocytes in a 96-well microtiter plate. The mixture was then incubated for 5 h at 30 °C. To measure the hemoglobin release, the erythrocytes were centrifuged (2000×*g*, 4 °C, 10 min), and the supernatants were assessed by measuring the absorbance at 540 nm using a spectrophotometer (Biotex, Vermont, USA). PBS was used as a non-hemolytic negative control and complete hemolysis was induced through osmotic shock with deionized water as a positive control. Each test was conducted in triplicates. The hemolytic percentage was calculated as follows:

$$\% \text{ Hemolysis} = 100 \times (OD_{540} \text{ of sample} - OD_{540} \text{ of negative control})/(OD_{540} \text{ of positive control} - OD_{540} \text{ of negative control}) \quad (2)$$

Effect of acid adaptation on *V. campbellii* virulence

Animal model

The virulence of acid-adapted and non-adapted *V. campbellii* HY01 was evaluated using wax moth larvae *G. mellonella*. Batches of *G. mellonella* larvae in their final instar stage were purchased from a local pet store. These were stored in the dark and were used within 7 days. All experiments used groups of 15 larvae weighing typically 250 mg each and each experiment was repeated using larvae from different batches [26].

Bacterial preparation

V. campbellii was grown in TSB-1.5, pH 7.3 (non-adapted) or TSB-1.5, pH 5.0 (acid-adapted) at 30 °C for 5 h. The cultured cells were harvested by centrifugation at 7600×*g* for 10 min, washed twice with PBS, and adjusted to 1.5×10^8 CFU/ml using a densitometer (Biosan, Riga, Latvia). 10-fold dilutions were also performed. The bacterial concentration was confirmed by a viable plate count method.

Infection experiment

For the evaluation of virulence, non-adapted cells of *V. campbellii* were injected into the insect larvae using 20 µl of bacterial suspension. A negative control group was injected with PBS only. The larvae were stored in petri dishes in the dark at room temperature for up to 48 h and observed every 24 h. The larvae were considered dead if they did not move in response to touch. The median lethal dose (LD_{50}) was then calculated [27]. The suitable concentration that caused 50 % of the insect larvae to die was used for virulence investigation of acid-adapted cells of *V. campbellii*. To assess whether the acid adaptation of *V. campbellii* would affect bacterial virulence *in vivo*, acid-adapted *V. campbellii* was used for larval injection. All experiments were carried out in triplicates.

Statistical analysis

For cell growth and hemolytic assay, the Duncan multiple range test was used to identify significant differences among the treatments. Larval mortality was compared using one-way ANOVA with SPSS version 14.0 (SPSS Inc., Illinois, USA). A P value of < 0.05 was considered to be statistically significant.

Results and discussion

Phylogenetic analysis of atypical hemolysin gene of *V. campbellii*

In this study, a 1,090 bp fragment of atypical hemolysin gene from *V. campbellii* was amplified and sequenced by PCR using gene-specific primers. Phylogenetic analysis showed that those atypical hemolysin gene sequences of *V. campbellii* isolates were almost identical (98 - 100 % similarity) (**Table 1**) and were grouped into the same cluster (**Figure 1**). The Blast analysis showed the similarity in hemolysin gene sequences among *V. campbellii* isolates, other vibrios, and *A. hydrophila* (**Table 1**). The hemolysin gene sequences of the *V. campbellii* strains showed 84 - 85 % similarity to the *hlyA* gene of *V. cholerae* and *V. harveyi* (**Table 1**). This indicates that the hemolysin gene may have been horizontally transferred from *V. cholerae* to *V. campbellii* and *V. harveyi*. The *hlyA* gene was ubiquitous among isolates of *V. cholerae*, suggesting that it may play an important role in the survival of this bacteria in its natural environment [28]. *Vibrio* spp. are native inhabitants of marine, brackish, and estuarine waters worldwide. The widespread occurrence of *V. cholerae* and other *Vibrio* species in the natural aquatic environment may lead to interspecies genetic exchange, as demonstrated by the presence of *hlyA* homologues in various *Vibrio* species, including *Vibrio anguillarum* (*vah1*), *Vibrio tubiashii* (*vthA* and *vthB*), *Vibrio coralliilyticus*, and *V. campbellii* [5,17,29-31]. Some strains of human pathogenic *V. fluvialis*, *V. mimicus*, and *Vibrio vulnificus* produce hemolysins that are similar to the HlyA of *V. cholerae* [32-34].

Table 1 Percent identity of hemolysin gene of *Vibrio campbellii* and that of other reference strains

Bacterial strain	Accession number	% identity (*E*-value) to			
		HY01	**PSU3282**	**PSU3283**	**PSU3288**
V. campbellii					
HY01	MF319496	100 (0.0)	98 (0.0)	100 (0.0)	99 (0.0)
PSU3282	MF319497	98 (0.0)	100 (0.0)	98 (0.0)	96 (0.0)
PSU3283	MF319498	100 (0.0)	98 (0.0)	100 (0.0)	99 (0.0)
PSU3288	MF319499	99 (0.0)	96 (0.0)	99 (0.0)	100 (0.0)
V. cholerae N16961	AE003853[a]	85 (0.0)	84 (0.0)	85 (0.0)	84 (0.0)
V. harveyi STD3-0949	GU230681[a]	85 (0.0)	85 (0.0)	85 (0.0)	84 (0.0)
V. harveyi STD3-0953	GU230682[a]	85 (0.0)	84 (0.0)	85 (0.0)	84 (0.0)
V. mimicus	GU137289[a]	74 (6e-133)	75 (4e-92)	74 (6e-133)	73 (1e-122)
V. fluvialis	AF348455[a]	71 (1e-93)	70 (2e-89)	71 (1e-93)	70 (3e-83)
V. anguillarum	LK021129[a]	63 (4e-10)	68 (3e-57)	63 (4e-10)	63 (4e-10)
Aeromonas hydrophila	U81555[a]	63 (3e-05)	65 (1e-04)	63 (3e-05)	63 (3e-05)

[a]Sequences of reference vibrio strains were obtained from NCBI.

Factors involved in hemolytic activity of *V. campbellii*

The link between hemolytic activity and virulence has been reported in both animal and human pathogenic vibrios. For example, hemorrhagic septicemia and diarrhea in fish infected with *V. anguillarum* has been suggested to associate with its hemolytic activity [13,31]. In addition, a mutation in the *vah1* hemolysin gene of *V. anguillarum* attenuated its virulence in fish [35]. *V. fluvialis* hemolysin is cytotoxic against the ovary cells of Chinese hamster, and is enterotoxic in sucking mice [33]. In this study, the laboratory conditions that could mimic the aquaculture environments were designed to investigate the hemolytic characteristics of *V. campbellii*. *V. campbellii* was able to grow in all conditions tested in this study (0.5 - 3.0 % NaCl, pH 5.0 - 8.6). However, the growth of *V. campbellii* was affected by salinity and pH. We demonstrated that the growth of *V. campbellii* was repressed at low salinity (0.5

% NaCl) and acidic pH (pH 5.0), while increasing either the salinity or the pH that enhanced the growth of *V. campbellii*. Furthermore, *V. campbellii* exhibited the highest growth rate when grown in TSB-1.5 or TSB-3, pH 8.6 (**Figure 2**). Our results are consistent with previously published observations that *V. campbellii* grew well in medium supplemented with 3 - 6 % NaCl. This suggests that this species was halophilic bacterium [36]. Moreover, *Vibrio* spp. are mostly found in saline marine environments where the pH is slightly alkaline. Thus, alkaline peptone water (APW) (1 % peptone, 1 % NaCl, pH 8.6±0.2) has long been used as a traditional enrichment broth for isolation of *Vibrio* spp. because the alkaline pH of the medium favors the growth of this genera [37].

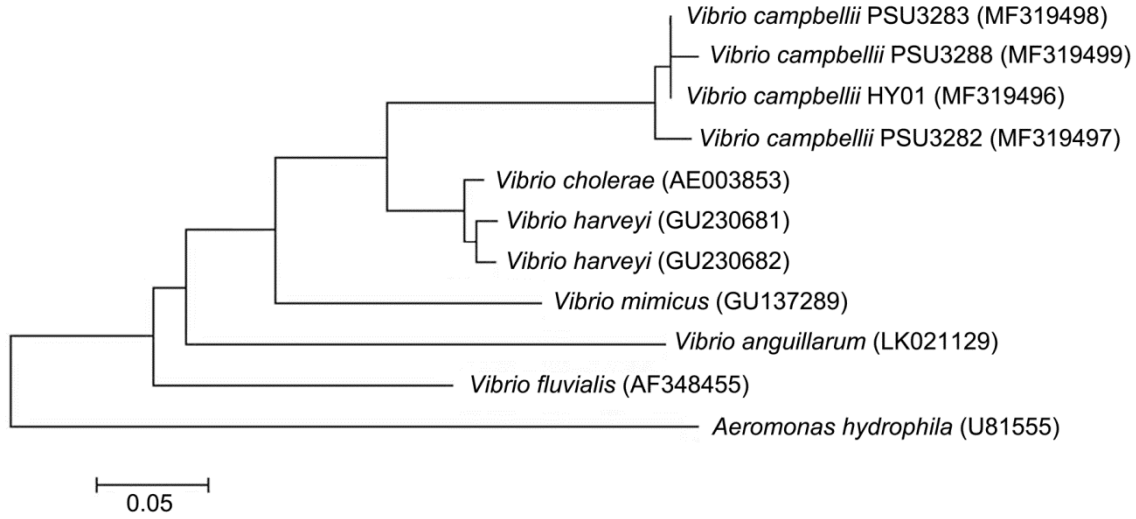

Figure 1 Phylogenetic analysis of atypical hemolysin gene of *Vibrio campbellii* and other reference species. Sequence information of reference strains was obtained from NCBI and the phylogeny was estimated using the neighbor-joining method with the program MEGA. The numbers indicated the bootstrap support from 1000 replicates.

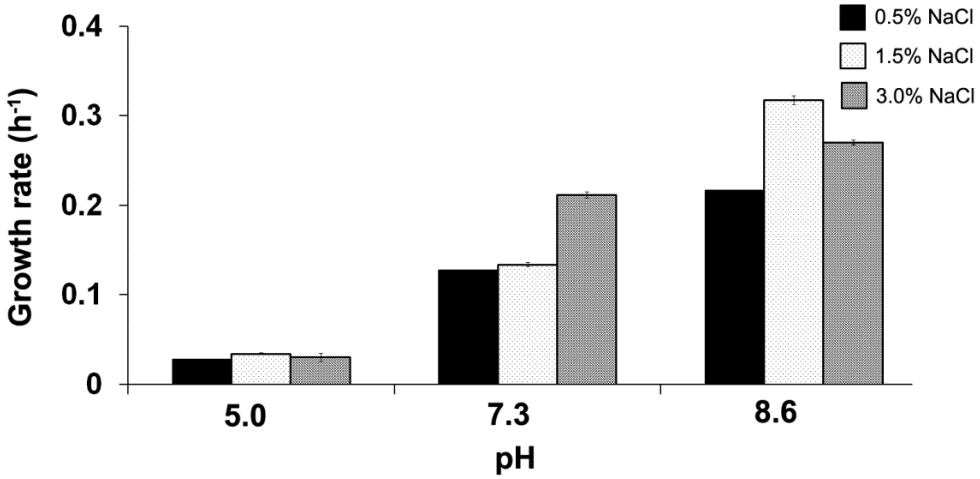

Figure 2 Effect of salinity and pH on growth of *Vibrio campbellii* HY01. Error bars indicate standard errors of the means

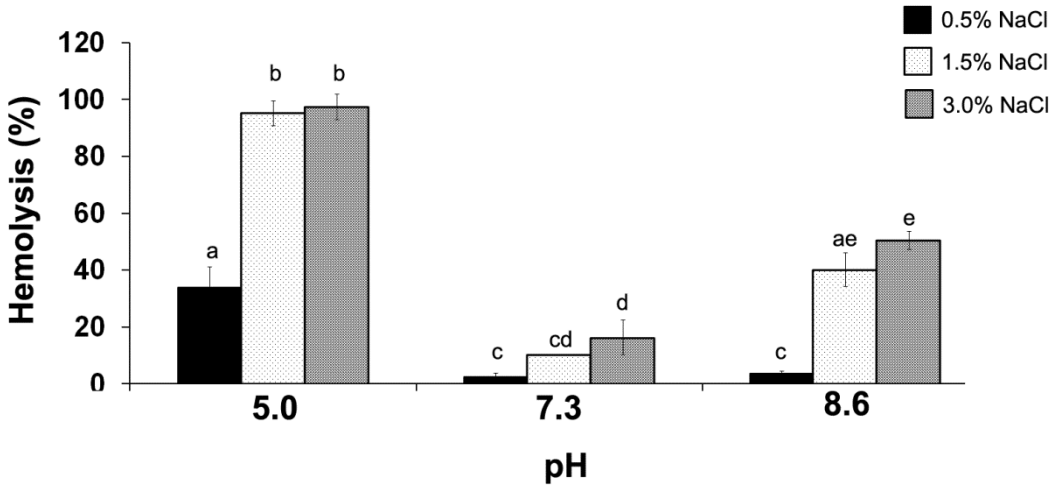

Figure 3 Effect of salinity and pH on hemolytic activity of *Vibrio campbellii* HY01. The error bars indicate standard errors of the means. Letters correspond to Duncan's multiple range test at 95 %.

In this study, we demonstrated that acid-adapted *V. campbellii* significantly exhibited higher hemolytic activity than non-adapted cells ($P < 0.05$). The hemolytic activity was also increased in cells previously grown at NaCl concentrations between 1.5 and 3.0 % (**Figure 3**). The hemolytic activity of *V. campbellii* has been reported as a strain dependent phenotype. This has been shown to be influenced by various factors, including cell envelop stress response [17,25]. Previous report has shown that hemolysin gene (*hlyA*) expression of *E. coli* O157:H7 was enhanced by prior exposure to the bacterium in acidic pH [38].

Effect of acid adaptation on *V. campbellii* virulence

In this work, *G. mellonella* was used as an infection model to assess *V. campbellii* virulence as it is a well characterized alternative host model for *V. anguillarum*, *V. cholerae,* and *V. parahaemolyticus* [26,39,40]. The mortality rates of 64.71, 9.09, and 2.86 % were observed in injected larvae after injection of *V. campbellii* HY01 at concentrations of 1.5×10^8, 1.5×10^7, and 1.5×10^6 CFU/ml, respectively (**Table 2**). Therefore, the LD_{50} of *V. campbellii* HY01 was calculated to be 8.16×10^7 CFU/ml. Inoculum concentrations equivalent to LD_{50} was used to analyze the effect of acid adaptation on *V. campbellii* virulence. The mortality of *G. mellonella* injected with acid-adapted *V. campbellii* at concentrations equivalent to 8.90×10^7 CFU/ml was 92.86 % (**Table 3**). The acid-adapted *V. campbellii* was considered more virulent (~20 times) against *G. mellonella* than the non-adapted *V. campbellii,* with an LD_{50} value of 4.33×10^6 CFU/ml for the acid-adapted *V. campbellii*. The effect of growth parameters on hemolytic activities were demonstrated in many bacterial pathogens, such as *Escherichia coli* and *Listeria monocytogenes* [41,42]. The bacteria which was adapted at low-pH conditions exhibited an increased virulence which has also been previously reported in *L. monocytogenes* [43]. The association of the increased in virulence and the hemolytic activity observed in this study needed further clarification. Changes in salinity and pH not only affect the virulence of the pathogens but also the susceptibility of the cultured animals to infectious diseases. In aquaculture, pH reduction would be occurring naturally during rainy season due to flushing of acid sulfate from soil into shrimp ponds [44].

Table 2 The LD_{50} test of *Vibrio campbellii* HY01 against *Galleria mellonella* larvae

***V. campbellii* concentration (CFU/ml)**	**No. of larvae dead/total**	**Cumulative no. of larvae**		**Mortality (%)**
		Dead	**Alive**	
1.5×10^8	9/15	11	6	64.71
1.5×10^7	1/15	2	20	9.09
1.5×10^6	1/15	1	34	2.86
1.5×10^5	0/15	0	49	0
Control (PBS)	0/15	NA[a]	NA[a]	0

[a]Not applicable.

Table 3 Mortality of *Galleria mellonella* larvae after injected with acid-adapted *Vibrio campbellii* HY01

***V. campbellii* concentration (CFU/ml)**	**No. of larvae dead/total**	**Cumulative no. of larvae**		**Mortality (%)**
		Dead	**Alive**	
8.9×10^7	13/15	26	2	92.86
8.9×10^6	11/15	13	6	68.42
8.9×10^5	2/15	2	19	9.52
Control (PBS)	0/15	NA[a]	NA[a]	0

[a]Not applicable.

Conclusions

In conclusion, apart from being the reservoir of virulence genes in the aquatic environment, the presence of the *V. cholerae hlyA* homologues in *V. campbellii* may serve to increase the bacterial fitness under specific environmental conditions. Vibriosis outbreaks depend primarily on the environmental factors. Thus, the disease may be controlled through careful maintenance of water salinity and pH at levels that may reduce bacterial growth and virulence. The results obtained would benefit shrimp industry in controlling emerging diseases in the foreseeable future.

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References

[1] T Sawabe, K Kita-Tsukamoto and FL Thompson. Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. *J. Bacteriol.* 2007; **189**, 7932-6.
[2] dlP Leobert D, CR Lavilla-Pitogo and MG Paner. Luminescent vibrios associated with mortality in pond-cultured shrimp *Penaeus monodon* in the Philippines: Species composition. *Fish Pathol.* 2001; **36**, 133-8.
[3] PA West, PR Brayton, TN Bryant and RR Colwell. Numerical taxonomy of vibrios isolated from aquatic environments. *Int. J. Syst. Bacteriol.* 1986; **36**, 531-43.
[4] B Gomez-Gil, S Soto-Rodriguez, A Garcia-Gasca, A Roque, R Vazquez-Juarez, FL Thompson and J Swings. Molecular identification of *Vibrio harveyi*-related isolates associated with diseased aquatic organisms. *Microbiology* 2004; **150**, 1769-77.

[5] P Rattanama, K Srinitiwarawong, JR Thompson, R Pomwised, K Supamattaya and V Vuddhakul. Shrimp pathogenicity, hemolysis, and the presence of hemolysin and TTSS genes in *Vibrio harveyi* isolated from Thailand. *Dis. Aquat. Org.* 2009; **86**, 113-22.
[6] FL Thompson, B Gomez-Gil, ATR Vasconcelos and T Sawabe. Multilocus sequence analysis reveals that *Vibrio harveyi* and *V. campbellii* are distinct species. *Appl. Environ. Microbiol.* 2007; **73**, 4279-85.
[7] M Hoffmann, SR Monday, M Fischer and EW Brown. Genetic and phylogenetic evidence for misidentification of *Vibrio* species within the Harveyi clade. *Lett. Appl. Microbiol.* 2012; **54**, 160-5.
[8] B Lin, Z Wang, AP Malanoski, EA O'Grady, CF Wimpee, V Vuddhakul, N Alves, FL Thompson, B Gomez-Gil and GJ Vora. Comparative genomic analyses identify the *Vibrio harveyi* genome sequenced strains BAA-1116 and HY01 as *Vibrio campbellii*. *Environ. Microbiol. Rep.* 2010; **2**, 81-9.
[9] L Wang, Y Chen, H Huang, Z Huang, H Chen and Z Shao. Isolation and identification of *Vibrio campbellii* as a bacterial pathogen for luminous vibriosis of *Litopenaeus vannamei*. *Aquacult. Res.* 2015; **46**, 395-404.
[10] X Dong, H Wang, G Xie, P Zou, C Guo, Y Liang and J Huang. An isolate of *Vibrio campbellii* carrying the *pirVP* gene causes acute hepatopancreatic necrosis disease. *Emerg. Microbes Infect.* 2017; **6**, e2.
[11] S Haldar, S Chatterjee, N Sugimoto, S Das, N Chowdhury, A Hinenoya, M Asakura and S Yamasaki. Identification of *Vibrio campbellii* isolated from diseased farm-shrimps from south India and establishment of its pathogenic potential in an Artemia model. *Microbiology* 2011; **157**, 179-88.
[12] M Nishibuchi, S Doke, S Toizumi, T Umeda, M Yoh and T Miwatani. Isolation from a coastal fish of *Vibrio hollisae* capable of producing a hemolysin similar to the thermostable direct hemolysin of *Vibrio parahaemolyticus*. *Appl. Environ. Microbiol.* 1988; **54**, 2144-6.
[13] C Rodkhum, I Hirono, JH Crosa and T Aoki. Four novel hemolysin genes of *Vibrio anguillarum* and their virulence to rainbow trout. *Microb. Pathog.* 2005; **39**, 109-19.
[14] Y Zhong, XH Zhang, J Chen, Z Chi, B Sun, Y Li and B Austin. Overexpression, purification, characterization, and pathogenicity of *Vibrio harveyi* hemolysin VHH. *Infect. Immun.* 2006; **74**, 6001-5.
[15] AK Bej, DP Patterson, CW Brasher, MC Vickery, DD Jones and CA Kaysner. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. *J. Microbiol. Meth.* 1999; **36**, 215-25.
[16] HN Cinar, M Kothary, AR Datta, BD Tall, R Sprando, K Bilecen, F Yildiz and B McCardell. *Vibrio cholerae* hemolysin is required for lethality, developmental delay, and intestinal vacuolation in *Caenorhabditis elegans*. *PLoS One* 2010; **5**, e11558.
[17] HAD Ruwandeepika, T Defoirdt, PP Bhowmick, M Shekar, P Bossier and I Karunasagar. Presence of typical and atypical virulence genes *in vibrio* isolates belonging to the Harveyi clade. *J. Appl. Microbiol.* 2010; **109**, 888-99.
[18] GRS Amaral, BSdO Silva, EO Santos, GM Dias, RM Lopes, RA Edwards, CC Thompson and FL Thompson. Genome sequence of the bacterioplanktonic, mixotrophic *Vibrio campbellii* strain PEL22A, isolated in the abrolhos bank. *J. Bacteriol.* 2012; **194**, 2759-60.
[19] S Selven and R Philip. Salinity a significant environmental factor for *Vibrio harveyi* virulence in *Fenneropenaeus indicus*. *Aquacult. Res.* 2013; **44**, 747-59.
[20] MA Bordas, MC Balebona, I Zorrilla, JJ Borrego and MA Morinigo. Kinetics of adhesion of selected fish-pathogenic *Vibrio* strains to skin mucus of gilt-head sea bream (*Sparus aurata* L.). *Appl. Environ. Microbiol.* 1996; **62**, 3650-4.
[21] SY Kim, SY Kim, SE Lee, SE Lee, YR Kim, YR Kim, CM Kim, CM Kim, PY Ryu, PY Ryu, HE Choy, HE Choy, SS Chung, SS Chung, JH Rhee and JH Rhee. Regulation of *Vibrio vulnificus* virulence by the LuxS quorum-sensing system. *Mol. Microbiol.* 2003; **48**, 1647-64.
[22] S Haldar, SB Neogi, K Kogure, S Chatterjee, N Chowdhury, A Hinenoya, M Asakura and S Yamasaki. Development of a haemolysin gene-based multiplex PCR for simultaneous detection of

Vibrio campbellii, *Vibrio harveyi* and *Vibrio parahaemolyticus*. *Lett. Appl. Microbiol.* 2010; **50**, 146-52.
[23] S Thaithongnum, P Ratanama, K Weeradechapol, A Sukhoom and V Vuddhakul. Detection of *Vibrio harveyi* in shrimp postlarvae and hatchery tank water by the Most Probable Number technique with PCR. *Aquaculture* 2006; **261**, 1-9.
[24] K Tamura, G Stecher, D Peterson, A Filipski and S Kumar. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 2013; **30**, 2725-9.
[25] P Rattanama, JR Thompson, N Kongkerd, K Srinitiwarawong, V Vuddhakul and JJ Mekalanos. Sigma E regulators control hemolytic activity and virulence in a shrimp pathogenic *Vibrio harveyi*. *PLoS One* 2012; **7**, e32523.
[26] T Nuidate, N Tansila, S Saengkerdsub, J Kongreung, D Bakkiyaraj and V Vuddhakul. Role of indole production on virulence of *Vibrio cholerae* using *Galleria mellonella* larvae Model. *Indian J. Microbiol.* 2016; **56**, 368-74.
[27] LJ Reed and H Muench. A simple method of estimating fifty per cent endpoints. *Am. J. Epidemiol.* 1938; **27**, 493-7.
[28] MH Brown and PA Manning. Haemolysin genes of *Vibrio cholerae*: presence of homologous DNA in non-haemolytic O1 and haemolytic non-O1 strains. *FEMS Microbiol. Lett.* 1985; **30**, 197-201.
[29] E de O Santos, N Alves Jr, GM Dias, AM Mazotto, A Vermelho, GJ Vora, B Wilson, VH Beltran, DG Bourne and F Le Roux. Genomic and proteomic analyses of the coral pathogen *Vibrio coralliilyticus* reveal a diverse virulence repertoire. *ISME J.* 2011; **5**, 1471-83.
[30] H Hasegawa, EJ Lind, MA Boin and CC Hse. The extracellular metalloprotease of *Vibrio tubiashii* is a major virulence factor for pacific oyster (*Crassostrea gigas*) larvae. *Appl. Environ. Microbiol.* 2008; **74**, 4101-10.
[31] I Hirono, T Masuda and T Aoki. Cloning and detection of the hemolysin gene of *Vibrio anguillarum*. *Microb. Pathog.* 1996; **21**, 173-82.
[32] GT Kim, JY Lee, SH Huh, JH Yu and IS Kong. Nucleotide sequence of the *vmhA* gene encoding hemolysin from *Vibrio mimicus*. *Biochim. Biophys. Acta* 1997; **1360**, 102-4.
[33] MH Kothary, H Lowman, BA McCardell and BD Tall. Purification and characterization of enterotoxigenic E1 Tor-like hemolysin produced by *Vibrio fluvialis*. *Infect. Immun.* 2003; **71**, 3213-20.
[34] K Yamamoto, AC Wright, JB Kaper and JG Morris. The cytolysin gene of *Vibrio vulnificus*: Sequence and relationship to the *Vibrio cholerae* El Tor hemolysin gene. *Infect. Immun.* 1990; **58**, 2706-9.
[35] JL Rock and DR Nelson. Identification and characterization of a hemolysin gene cluster *in Vibrio anguillarum*. *Infect. Immun*. 2006; **74**, 2777-86.
[36] S Haldar, S Chatterjee, N Sugimoto, S Das, N Chowdhury, A Hinenoya, MA sakura and S Yamasaki. Identification of *Vibrio campbellii* isolated from diseased farm-shrimps from south India and establishment of its pathogenic potential in an Artemia model. *Microbiology* 2011; **157**, 179-88.
[37] AL Furniss, JV Lee and TJ Donovan. *The Vibrios*. London, 1978, p. viii-58.
[38] D Elhanafi, B Leenanon, W Bang and MA Drake. Impact of cold and cold-acid stress on poststress tolerance and virulence factor expression of *Escherichia coli* O157: H7. *J. Food Prot*. 2004; **67**, 19-26.
[39] S McMillan, D Verner-Jeffreys, J Weeks, B Austin and AP Desbois. Larva of the greater wax moth, *Galleria mellonella*, is a suitable alternative host for studying virulence of fish pathogenic *Vibrio anguillarum*. *BMC Microbiol.* 2015; **15**, 127.
[40] S Wagley, R Borne, J Harrison, C Baker-Austin, D Ottaviani, F Leoni, V Vuddhakul, and RW Titball. *Galleria mellonella* as an infection model to investigate virulence of *Vibrio parahaemolyticus*. *Virulence* 2018; **9**, 197-207.
[41] A Bavdek, R Kostanjek, V Antonini and JHa Lakey. PH dependence of listeriolysin O aggregation and pore-forming ability. *FEBS J.* 2012; **279**, 126-41.

[42] H Schmidt, E Maier, H Karch and R Benz. Pore-forming properties of the plasmid-encoded hemolysin of enterohemorrhagic *Escherichia coli* O157:H7. *Eur. J. Biochem.* 1996; **241**, 594-601.
[43] B O'Driscoll, CG Gahan and C Hill. Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. *Appl. Environ. Microbiol*. 1996; **62**, 1693-8.
[44] D Johnston and Ma Lourey. Water quality and plankton densities in mixed shrimp-mangrove forestry farming systems in Vietnam. *Aquacult. Res.* 2002; **33**, 785-98.