Real-time PCR Quantification of White Spot Syndrome Virus (WSSV) and Hepatopancreatic Parvovirus (HPV) Loads in Shrimp and Seawaters of Shrimp Ponds on the West Coast of South Korea

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Viral diseases are major emerging problems of shrimp that have affected the production, and even complete losses for shrimp farms. In this study, we developed a sensitive TaqMan real-time PCR method to quantify white spot syndrome virus (WSSV) and hepatopancreatic parvovirus (HPV) in the shrimp and pond water in which fleshy shrimp, *Fenneropenaeus chinensis*, and Pacific white shrimp, *Litopenaeus vannamei*, are reared. WSSV and HPV in pond seawaters ranged from 1.65×10^3 to 2.43×10^9 and from 0 to 4.43×10^5 copies/L of seawater, respectively. Of 20 ponds analyzed, all pond water and shrimp were positive for WSSV. *L. vannamei* showed higher susceptibility to WSSV than *F. chinensis*. HPV was detected only in the pond water for *F. chinensis*. In shrimp tissue, however, HPV was found in both species, with 23-times higher infection rate in *F. chinensis* than *L. vannamei*. The total bacterial counts in the pond water ranged from 2.23×10^3 to 1.98×10^5 CFU/mL. The variations in total bacterial count for each pond appeared to correlate to the variations of the WSSV load. Statistical analysis indicated that there was no significant difference ($P > 0.05$) between the WSSV load in pond water and shrimp, and there was no relationship between total bacterial load and viral load in the pond water. However, a significant difference ($P < 0.01$) was found between HPV load and *L. vannamei* and *F. chinensis* pond water.

Key words: TaqMan real-time PCR, White spot syndrome virus, Hepatopancreatic parvovirus, *Fenneropenaeus chinensis*, *Litopenaeus vannamei*

Introduction

Viral diseases are major problems in the shrimp aquaculture industry worldwide, as serious viral outbreaks often cause disastrous losses in shrimp farming (Lightner et al., 1996). White spot syndrome virus (WSSV) and hepatopancreatic parvovirus (HPV) are the most prevalent viral diseases affecting the shrimp in the Yellow Sea (Pantoja et al., 2005). Fifty five percent of shrimp farms in South Korea experienced total or partial losses of farmed shrimp because of these two viruses (Jang et al., 2006).

Since 1992, WSSV has caused catastrophic losses in cultured shrimp populations (Chen, 1995; Chou et al., 1995; Wang et al., 1995; Lightner, 1996; Flegel, 1997). This virus can cause 100% mortality in in-

fecting shrimp within 3 to 5 days (Nakano et al., 1994; Lightner, 1996). In South Korea, it first occurred in 1993 in ponds of *Marsupenaeus japonicus* on the west coast and has caused great economic losses to the shrimp culture industry every year (Kim et al., 1997; Heo et al., 2000; Jang et al., 2007a; b). For 2004 in particular, 32.9% of the shrimp farms in the country collapsed due to direct or indirect causes associated with WSSV (Jang et al., 2007b; Jang et al., 2008).

HPV is also an emerging disease, which was first detected in 1984 in wild *Fenneropenaeus merguensis* and *F. indicus* from Singapore (Chong and Loh, 1984). During outbreaks, mortalities attributed to HPV have reached 100% (Lightner, 1996; Flegel et al., 1999; Pantoja et al., 2005). Its current geographic range is from East Africa to Korea, including

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Australia and the Pacific, and the Atlantic coasts of the Americas (Lightner, 1996). The first Korean record of HPV came from samples of *F. chinensis* in 1991 (Park, 1992; Jang and Jun, 2005).

Since 2003, the native species, *F. chinensis* and *M. japonicus*, have been replaced with the Pacific white shrimp, *Litopenaeus vannamei*, due to the damage to shrimp farming associated with WSSV and HPV (Jang et al., 2006). In 2007, the farmed production of *L. vannamei* accounted for about 62.5% of the total farmed shrimp production in Korea (Jang, unpublished data). Apart from the viral diseases, shrimp farms were also facing bacterial disease problems, such as vibriosis, which causes high mortalities in penaeid shrimp culturing systems (Lightner and Lewis, 1975; Takahashi et al., 1985; Lightner, 1988; Brock and Lea-Master, 1992; Mohny and Lightner, 1994).

The polymerase chain reaction (PCR) has been used worldwide for diagnoses of shrimp viruses (Lightner and Redman, 1998; Pantoja and Lightner, 2001). However, there remain limitations for conventional PCR methods, as they can only detect the presence of virus, but cannot quantify the extent of viral infection. Real-time PCR, an alternative method to conventional PCR, is capable of providing a precise quantitative measure of the number of viral copies in a sample. Real-time PCR has been increasingly successfully used to quantify several viral pathogens, including WSSV and HPV, of different shrimp species, including *Penaeus monodon*, *P. merguensis*, *L. vannamei*, *L. stylirostris* and *F. chinensis* (Tang and Lightner, 2000; Durand et al., 2003; Kathy et al., 2006; Jang et al., 2008).

Experience has also shown that shrimps infected with WSSV and HPV could survive for the entire culture period without clinical signs by manipulating environmental factors (Lightner and Redman, 1998). It is likely that shrimp infected with WSSV may not develop the characteristic clinical signs under certain environmental and physiological conditions, as asymptomatic carriers and shrimp with low levels of infection may not develop typical white spots (Liu et al., 2006). Durand and Lightner (2002) and Durand et al. (2003) reported that juveniles of artificially infected *L. stylirostris* and *L. vannamei*, with 1.2×10^4 to 3.0×10^7 WSSV copies ng^{-1} of DNA, showed clinical signs of the disease or had moribund behaviors. However, the number of studies on the relationship between WSSV infection and pond water is very limited (Song et al., 2008).

The present study quantified WSSV and HPV loads in shrimps and their rearing seawater on the

west coast of South Korea using highly sensitive and specific TaqMan real-time PCR. We also analyzed the correspondence of viral status with *Vibrio* bacteria and environmental parameters.

Materials and Methods

Sample collection

During the period from July 22 to August 7, 2008, shrimps were collected from 17 ponds for *L. vannamei* and 3 ponds for *F. chinensis* along the west coast of South Korea (Fig. 1 and Table 4). Pleopods were removed from 5 animals in each pond and preserved separately in 70% ethanol for DNA extractions. For all 20 ponds, 1 L water samples were collected in plastic bags for processing. Natural seawater was also collected from the coast of Taean, Chungnam Pro-vince as a control.

Fig. 1. Geographical locations of sampling sites on the west coast of South Korea.

Water quality analysis

Water quality parameters, including dissolved oxygen (DO), temperature, pH and salinity, were measured using an YSI meter (model 610, Yellow Spring Instrument Co., Ohio, USA). Ammonium-N, nitrite-N and alkalinity concentrations were measured at the site using a colorimetric kit (Merck Co., Germany).

Enumeration of bacteria

Pond water samples of 1 mL were serially diluted to 10^{-5} in 1% sterile saline and 100 μ L aliquots of each dilution were spread plated onto 2 media, marine agar (MA; Difco, USA) and *Vibrio*-specific thiosulfate citrate bile salt sucrose (TCBS) agar. Duplicate plates were prepared for each dilution. Hepatopancreas and hemolymph of the shrimp were directly streaked onto TCBS agar using a sterile disposable inoculation loop for qualitative assay. All plates were incubated for 24 to 42 hrs at $30 \pm 2^\circ\text{C}$ prior to colony enumeration. Plates containing 30-300 colonies/mL were used for bacterial counts.

Processing of water samples for viral concentrations

Water samples were processed following published protocols (Katayama et al., 2002; Song et al., 2008). Water samples of 500 mL from each pond were filtered using a vacuum pump with type GF/F 1.2 μ m (Whatman International Ltd., UK) and type HA 0.45 μ m (Millipore Ltd., Burlington, MA, USA), 47 mm diameter negatively charged membrane filters. The filters were washed with 50 mL of 0.5 mM H_2SO_4 to remove cations, and viruses were eluted from the filter with 1.25 mL of elution buffer (1 mM NaOH), followed by neutralizer (6.25 mL of 50 mM H_2SO_4 and 6.25 mL of 100X Tris EDTA buffer). The filtrate was then concentrated to 200 μ L by centrifuging the samples in a vivaspin concentrator (Sartorius Ltd., UK) at $15,000 \times g$ for 10 min.

DNA Extraction

Total genomic DNA and associated viral DNA were extracted from about 25 mg pleopod and 300 μ L processed water samples using DNeasy Kits (Qiagen Ltd., CA, USA). DNA concentration and quality were determined by measuring optical densities at OD_{260} nm and OD_{280} nm with a spectrophotometer, Genesys 5 (Spectronic Instrument Inc., New York, USA).

Quantitative real-time PCR

Real-time PCR was performed using a total volume of 20 μ L, containing 70 ng of total DNA, 1X

Perfect Real Time premix (RR039A, Takara, Japan). For WSSV quantification, 0.125 μ M TaqMan probe and 0.25 μ M of each WSSV specific primer (Table 1) and for HPV quantification 0.25 μ M of each HPV specific primer and 0.1 μ M TaqMan probe (Table 1) were used in the total volume. Thermal cycling consisted of an initial denaturation step at 95°C for 30 s, followed by 45 cycles of 60°C for 5 s annealing and 20 s extension steps on the Rotor Gene 6000 (Corbett Research Inc., Australia). The quantity of each sample was determined using Rotor Gene Operating Software version 1.7.61. Each sample was tested in triplicate. The copy number of the target amplicon in the plasmid was estimated and 10-fold serial dilutions were made for use as absolute standards for quantification. The viral copy number was normalized on a nanogram (ng) genomic DNA basis, or on an mL basis for water. For each new run, at least 2 NTCs (non-template control) were performed as negative controls.

Statistical analysis

SPSS 16 statistical software (available at <http://www.spss.com>) was used to analyze the differences of copy numbers between *F. chinensis* and *L. vannamei* culturing ponds for WSSV and HPV loads in shrimp and pond water, and between viral loads and total bacterial loads.

Results

Specificity and sensitivity of the TaqMan real-time PCR

Specificity tests showed that there were no cross-reactions between shrimp DNA and other crustacean viral DNA. For 2 independently generated standard curves, linear relationships were observed for the input copy numbers of the virus template, with high reaction efficiencies and appropriate slopes (Fig. 2, Fig. 3). Mean CT values for the virus-specific products were obtained over six $\log_{10}$ dilutions, ranging from 5.9×10^0 copies ($\text{CT} = 41.26 \pm 0.27$) to 5.9×10^6 copies ($\text{CT} = 21.34 \pm 0.23$) for WSSV (Table 2), and 4.37×10^0 copies ($\text{CT} = 39.26 \pm 0.45$) to 4.37×10^6

Table 1. Sequences of primers and probe used for real-time PCR

Name	Sequence(5'→3')	Size
RT-WSSV-F ₁₅₄	CCAGTTCAGAATCGGACGTT	154
RT-WSSV-R ₁₅₄	AAAGACGCCTACCCTGTTGA	
WSSV Probe	FAM-TCCATAGTTCCTGGTTTGTAAATGTGCCG-BHQ	
RT-HPV-F ₁₁₉	CTAGCATGGGAGCAGTCGTA	119
RT-HPV-R ₁₁₉	GACCTGAACAGTCTCTGCCA	
HPV Probe	FAM-TCCATAGTTCCTGGTTTGTAAATGTGCC-BHQ

Fig. 2. Standard curve for WSSV TaqMan real-time PCR. Threshold cycles (CT) plotted against WSSV concentration. R, correlation coefficient; M, slope; B, intercept; Efficiency, PCR amplification efficiency.

Fig. 3. Standard curve for HPV TaqMan real-time PCR. Threshold cycles (CT) plotted against HPV concentration. R, correlation coefficient; M, slope; B, intercept; Efficiency, PCR amplification efficiency.

Table 2. Inter- and intra-assay reproducibilities for 4 independent assays (2 replicates for each assay) of the TaqMan real-time PCR for WSSV

Assay	Ct values at different WSSV copy number						
	5.90×10^6	5.90×10^5	5.90×10^4	5.90×10^3	5.90×10^2	5.90×10^1	5.90×10^0
Mean	21.34	24.84	28.18	31.32	34.91	38.43	41.26
S.D. ^a	0.23	0.24	0.09	0.36	0.42	0.47	0.27
CV (%) ^b	1.08	0.96	0.32	1.14	1.19	1.21	0.65

^astandard deviation; ^bcoefficient of variance; ^cthreshold cycle.

Table 3. Inter- and intra-assay reproducibilities for 4 independent assays (2 replicates for each assay) of the TaqMan real-time PCR for HPV

Assay	Ct values at different HPV copy number						
	4.75×10^6	4.75×10^5	4.75×10^4	4.75×10^3	4.75×10^2	4.75×10^1	4.75×10^0
Mean	19.13	22.67	25.49	28.91	32.14	35.65	39.26
S.D. ^a	0.09	0.11	0.06	0.09	0.45	0.32	0.45
CV (%) ^b	0.005	0.002	0.002	0.001	0.17	0.03	0.17

^astandard deviation; ^bcoefficient of variance; ^cthreshold cycle.

copies (CT=19.13±0.09) for HPV (Table 3). Successful amplifications were obtained from 10^9 down

to 2 copies for both viruses using optimal PCR reaction mixtures. Negative controls did not display

any amplification for either virus.

TaqMan real-time PCR quantification of WSSV and HPV loads in pond water and shrimp

To measure the WSSV and HPV loads in pond waters and shrimp, the CT value of each sample was interpreted from the standard curve for the corresponding virus. Viral loads in the pond waters varied from 1.65 to 2.43×10^6 and from 0 to 4.43×10^2 copies/mL for WSSV (Fig. 4) and HPV (Fig. 5), respectively. The copies present in an ng of DNA from shrimp tissue ranged from 0.69 to 3.99×10^6 and from 0 to 1.79×10^2 for WSSV and HPV, respectively. Statistically, there was no significant difference ($P > 0.05$) for the mean copy numbers of the shrimp and pond water for WSSV. However, there was a significant difference ($P < 0.01$) between the pond water of *L. vannamei* and *F. chinensis* for HPV. WSSV was detected in control seawater with a mean of 1.15×10^1 copies/mL.

Bacterial counts

Total bacterial counts ranged from 2.23×10^3 to 1.98×10^5 CFU/mL and from 2.8×10^3 to 1.05×10^5 CFU/mL in *L. vannamei* and *F. chinensis* pond water, respectively (Table 5). The *Vibrio* counts obtained from water samples ranged from zero to 6.40×10^2 CFU/mL. The *Vibrio* status in hepatopancrease and haemolymph samples of shrimp is summarized in Table 5. There was no significant difference ($P > 0.05$) observed between the viral load and total bacterial count.

Selected water quality parameters

Temperatures of pond water ranged from 26.3 – 29.5°C , $\text{NH}_3\text{-N}$ ranged from 0 – 0.56 ppm and $\text{NO}_2\text{-N}$ ranged from 0 – 0.20 ppm (Table 6). In pond 7A, most of the parameters were abnormal, including high alkalinity (150 ppm), $\text{NH}_3\text{-N}$ (0.1 ppm), temperature (28.4°C) and salinity (29.6 psu).

Fig. 4. WSSV load in shrimp rearing water and shrimp collected from shrimp ponds on the west coast of Korea from Jul. to Aug., 2008.

Fig. 5. HPV load in shrimp rearing water and shrimp collected from shrimp ponds on the west coast of Korea from Jul. to Aug., 2008.

Table 4. Mean values for viral loads in rearing water and shrimp, *F. chinensis* and *L. vannamei* collected from shrimp ponds on the west coast of Korea from Jul. to Aug., 2008

Pond	Location	Date of Sample collection	Mean body weight (g)	Species	WSSV		HPV	
					copies/ng DNA in shrimp tissue	copies/mL in water	copies/ng DNA in shrimp tissue	copies/mL in water
5A	Taeon	Jul. 22	4.5	<i>L. vannamei</i>	0.92×10^1	2.17×10^1	2.39×10^1	0
7A	Taeon	Jul. 22	3.4	<i>L. vannamei</i>	1.27×10^6	2.43×10^6	3.80×10^1	0
8B	Taeon	Jul. 23	5.1	<i>L. vannamei</i>	1.25×10^1	5.00×10^1	0.67×10^1	0
9A	Taeon	Jul. 23	6.2	<i>L. vannamei</i>	0.64×10^1	9.85×10^1	0	0
9B	Taeon	Jul. 23	4.5	<i>L. vannamei</i>	0.15×10^1	3.91×10^1	0	0
11A	Taeon	Jul. 23	8.9	<i>L. vannamei</i>	0.15×10^1	4.12×10^2	0	0
12A	Taeon	Jul. 23	7.5	<i>L. vannamei</i>	0.06×10^1	1.19×10^2	0	0
13A	Boryeong	Jul. 25	7.1	<i>L. vannamei</i>	0.90×10^1	2.56×10^1	0	0
22A	Ganghwa	Jul. 29	3	<i>L. vannamei</i>	1.47×10^1	0.39×10^1	0	0
23A	Ganghwa	Jul. 29	5.9	<i>L. vannamei</i>	1.16×10^1	0.33×10^1	0	0
24A	Hwaseong	Jul. 31	7.6	<i>L. vannamei</i>	2.45×10^1	0.42×10^1	0	0
26B	Hwaseong	Jul. 31	3.1	<i>L. vannamei</i>	4.09×10^1	4.74×10^3	0	0
28G	Ansan	Jul. 31	2.9	<i>L. vannamei</i>	3.99×10^6	5.78×10^5	0	0
29A	Gochang	Aug. 4	7.1	<i>L. vannamei</i>	0.26×10^1	7.04×10^1	0	0
42A	Haenam	Aug. 7	10	<i>L. vannamei</i>	0.43×10^1	0.16×10^1	0.04×10^1	0
108A	Sinan	Aug. 6	12.2	<i>L. vannamei</i>	0.30×10^1	0.41×10^1	0	0
109A	Sinan	Aug. 6	1.7	<i>L. vannamei</i>	1.80×10^4	5.06×10^4	0	0
15AI	Ganghwa	Jul. 28	4.4	<i>F. chinensis</i>	0.39×10^1	1.13×10^1	0.37×10^1	0
17AI	Ganghwa	Jul. 28	2.8	<i>F. chinensis</i>	0.58×10^1	7.06×10^1	1.01×10^2	5.12×10^1
41A	Haenam	Aug. 7	3	<i>F. chinensis</i>	3.47×10^1	3.58×10^1	1.79×10^2	4.43×10^2
Total mean					2.64×10^5	1.50×10^5	1.76×10^1	2.47×10^1
Mean for <i>L. vannamei</i>					3.10×10^5	1.77×10^5	0.40×10^1	0
Mean for <i>F. chinensis</i>					1.48×10^1	3.92×10^1	9.47×10^1	1.64×10^2
Control seawater*					-	1.15×10^1	-	0

* Seawater collected from coast of Taeon.

Table 5. Bacterial counts in pond water and presence of *Vibrio* in shrimp collected from shrimp ponds on the west coast of Korea from Jul. to Aug., 2008

Pond	Pond water		Number of <i>Vibrio</i> - positive in shrimp*	
	Total bacterial count (CFU/mL)	<i>Vibrio</i> count (CFU/mL)	Hepatopancrease	Haemolymph
5A	1.98×10^5	160	3/3	1/3
7A	3.80×10^3	640	1/3	0/3
8B	1.68×10^4	180	1/3	0/3
9A	6.53×10^4	ND	0/3	0/3
9B	2.62×10^4	60	13	0/3
11A	4.37×10^4	ND	1/3	0/3
12A	1.93×10^5	ND	0/3	0/3
13A	1.90×10^4	20	0/3	0/3
22A	1.51×10^5	60	3/3	0/3
23A	2.33×10^3	ND	2/5	0/5
24A	4.43×10^4	ND	0/5	0/5
26B	7.27×10^3	ND	2/5	0/5
28G	2.31×10^4	ND	3/3	3/3
29A	1.04×10^5	20	1/3	0/3
42A	1.13×10^4	ND	0/3	0/3
108A	4.20×10^3	ND	1/3	0/3
109A	1.20×10^4	440	2/4	0/4
15AI	1.05×10^5	ND	0/5	0/5
17AI	2.80×10^3	40	0/5	0/5
41A	8.60×10^4	50	1/5	0/5

ND, not determined; *, number of *Vibrio*- positive/total number of shrimp assayed.

Table 6. Selected water quality parameters in indicated pond water

Pond	Temperature (°C)	Salinity (psu)	pH	DO (mg/mL)	Alkalinity (mg/mL)	NH ₃ -N (mg/mL)	NO ₂ -N
5A	28.0	29.2	ND	6.86	135	0.05	0.001
7A	28.4	29.6	ND	5.90	150	0.10	0.03
8B	27.2	31.0	8.24	6.60	140	0.03	0.20
9A	26.3	27.9	ND	6.36	140	0.06	0
9B	26.7	28.3	ND	5.67	145	0.38	0
11A	26.7	27.1	8.20	7.20	130	0	0.05
12A	27.0	27.0	8.20	6.05	135	0	0
13A	26.3	27.7	8.00	5.53	135	0.09	0.05
22A	ND	ND	8.70	ND	80	0.01	0
23A	ND	ND	8.50	ND	95	0.12	0.02
24A	28.8	9.0	7.60	5.86	150	0.56	0.20
26B	29.0	23.7	8.00	6.48	150	0.26	1.00
28G	28.2	25.2	8.50	4.84	90	0.06	0.01
29A	28.8	22.7	8.00	7.76	110	0.03	0.02
42A	29.3	27.5	8.60	8.00	150	0.27	0.02
108A	ND	ND	7.50	ND	125	0.02	0.01
109A	ND	ND	8.50	ND	140	0.06	0.15
15AI	ND	ND	8.00	ND	90	0.02	0.02
17AI	ND	ND	8.20	ND	150	0.04	0.15
41A	29.5	32.2	7.00	ND	125	0.02	0.05

ND, no data.

Discussion

Viral diseases in shrimp aquaculture have been a challenge due to a lack of highly sensitive detection methods. Even though PCR and nested PCR are more sensitive and convenient than other diagnostic methods (Lo et al., 1996), they cannot detect the presence of a single copy of viral genome in infected tissue. Over recent years, the highly sensitive and specific real time PCR has been developed and used for detecting viral disease due to its amenability to high throughput screening (Durand and Lightner, 2002; Durand et al., 2003; Kathy et al., 2007). In the present study, we established sensitive and reliable TaqMan real-time PCR techniques for WSSV and HPV for *F. chinensis* and *L. vannamei*; this could detect 2 to 10⁹ target copies per reaction. The sensitivity of this method enabled us to assay shrimp tissue samples bearing very low WSSV and HPV infection loads, with 1.5 and 0.45 copies/ng of genomic DNA, respectively (Table 4).

In an aquaculture facility, the most probable common pathway for pathogen introduction is via water (Lotz and Lightner, 1999). Hence, it is important to monitor the viral loads in rearing waters. In this study, the results suggest that there were no significant variations in the WSSV loads of shrimp and water, with means of 2.64×10⁵ copies/ng DNA for shrimp tissue and 4.93×10⁵ copies/mL for water (Fig. 4). The abundance of WSSV in *F. chinensis* rearing water, ranging from 1.13×10⁴ to 7.06×10⁴ copies/L, is con-

sistent with the results of Song et al. (2008). They found 3.8×10³-1.2×10⁵ WSSV copies/L in rearing waters for *F. chinensis* on the west coast of South Korea. In the control seawater for the present study, WSSV was found at 1.15×10⁴ copies/L. This suggests that free WSSV is present in natural seawater (Chou et al., 1998; Song et al., 2008).

HPV was not detected in any rearing water for *L. vannamei* ponds, but it was found for *F. chinensis* pond water (Fig. 5). Moreover, for *F. chinensis*, the HPV infection rate was 23-times higher than for *L. vannamei* in tissue samples. This result may indicate that *L. vannamei* is either not susceptible or is more resistant to HPV than *F. chinensis*. In contrast, the WSSV infection rate was much higher in *L. vannamei* than *F. chinensis*. This suggests that *L. vannamei* is more resistant to WSSV than other shrimp (Wyban and Sweeny, 1991), although *L. vannamei* is, in fact, highly susceptible to WSSV and TSV (Taura syndrome virus) and it is a carrier of IHHNV (infectious hypodermal and hematopoietic necrosis virus) and LOVV (lymphoid organ vacuolization virus) (Briggs et al., 2004).

In addition to the viral diseases, diseases caused by bacteria are considered economically significant disease agents of shrimp (Lightner and Lewis, 1975; Lightner et al., 1994). Infections due to *Vibrio* spp. affect shrimp in hatcheries and grow-out facilities, causing chronic or mass mortalities. Although bacteria are easily isolated, the mechanisms for their

pathogenicity remain unclear (Lavilla-Pitogo, 1996). Thus, it is necessary to isolate bacteria. It was noted in our study that the variations in the total bacterial counts from each pond appeared to correlate to the variations of WSSV loads, although statistical analysis indicated that there was no significant relationship ($P>0.05$) between total bacterial loads and viral loads in the pond water. However, for pond 7A in particular, the *Vibrio* count was increased in the water and hepatopancreas samples of shrimp also showed positive with respect to increased WSSV load (Table 5). To understand the relationship between the bacteria and virus, further studies are needed.

With respect to water quality parameters, viral abundance is related to interactions between shrimp and changes of environmental factors (Table 6). Previous studies have focused upon relationships between shrimp and their environmental parameters (Liu et al., 2006; Song et al., 2008). With regard to water, there was an indication for poor quality ranges of units in all the ponds that showed high viral loads (Table 6). The water quality parameters of pH, DO, temperature and N-compounds, including NH_3 and NO_2 , are very decisive factors in shrimp culture. Environmental factors are directly related to immune responses of shrimp in marine shrimp culture (Bray and Lawrence, 1993; Cheng and Chen, 2000). In the present study, however, it was very difficult to find any relationship between the environmental parameters and viral loads due to a lack of long-term monitoring.

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