Establishment of Threshold Infection Levels of WSSV in Different Weight Ranges of *Penaeus vannamei* Using Quantitative PCR (qPCR)

Leobert D. de la Peña, Joey I. Arboleda, and Jose Louis A. Castellano
Aquaculture Department, Southeast Asian Fisheries Development Center (SEAFDEC/AQD), Tigbauan, Iloilo 5021, Philippines
leobertd@seafdec.org.ph

Abstract

Threshold infection level is the pathogen load of the test animals measured before the appearance of clinical signs and mortality. This study aims to establish the threshold infection levels of WSSV in different weight ranges of *Penaeus vannamei* using qPCR. Artificial infection experiments were conducted using four weight ranges (3–5 g, 7–8 g, 15–18 g, and 22–25 g). The LD_{50} of the different weight ranges of shrimps were achieved at viral dilution of 10^{-6} and 10^{-5} after 216–240 hpi, and the viral loads of these inoculums have a range of 10^5–10^6 WSSV DNA copies/g. The viral loads of the samples in the time-course infection experiments when the mortalities started was determined at 10^9 WSSV DNA copies/g, while for the survivors was at 10^6 WSSV DNA copies/g. The threshold infection level of WSSV in shrimp was determined at 10^7 to 10^8 WSSV DNA copies/g. It was also found out that the threshold infection level was not weight dependent.

Keywords: Threshold infection levels, White Spot Syndrome Virus, Penaeus vannamei, Quantitative PCR

Introduction

Viral diseases have caused major constraints in shrimp farming in most Asian countries and the world. According to the OIE List of Crustacean Diseases for 2020, White Spot Syndrome Virus (WSSV) is still included since its first emergence and outbreaks in the early 1990s from China, Japan, Taiwan, and northern Thailand, and was subsequently spread into southern Thailand, India, and Indonesia (Lotz, 1997). Experimental evidence suggests that WSSV can be transferred horizontally and vertically through water, carrier organism, cannibalism, or infected broodstock (Flegel, 1997; de la Peña *et al*., 2007; Hoa *et al*., 2011). Approximately 3–10 days following onset of infection, mortality rates typically reach levels greater than 80% and usually 100 % of the population (Nakano *et al*., 1994; Kasornchandra *et al*., 1996; Lightner, 1996).

WSSV was first detected in the Philippines in early 1999 using PCR and Western blot assays in different life stages of cultured *Penaeus monodon*. Moreover, out of the

71 samples analyzed, there were 51 (72 %) were WSSV-positive (both for 1-step and 2-step, non-nested PCR) (Magbanua *et al*., 2000). Prevalence of WSSV in wild-caught *P. monodon* was also reported in the different sampling sites in the Philippines (de la Peña *et al*., 2007). Collection of these contaminated, wild *P. monodon* as spawners or broodstocks could serve as the primary source of WSSV contamination in shrimp farms due to vertical transmission of the virus in hatcheries (de la Peña *et al*., 2007).

The continued outbreaks of viral diseases necessitate the aquaculture industry to establish preventive and control measures to mitigate its negative impacts in production both in the hatchery and grow-out phases. There have been several experimental runs on developing novel vaccines and chemotherapeutants to date. However, there were no consistently effective methods (OIE, 2019). The majority of the shrimp farms adopted the domestication of specific pathogen-free (SPF) shrimp stocks. Other prevention and control methods provide immunostimulants and probiotics in the grow-out phase and practice disinfection of broodstock or spawners and eggs. Another emerging approach is breeding specific pathogen-resistant (SPR) shrimp stocks (Cuellar-Anjel *et al*., 2012; Huang *et al*., 2011).

On the other hand, pro-active monitoring and early detection of devastating pathogens are the most efficient responses so that immediate and appropriate interventions to prevent and control the spread of infection can be implemented. The main objective of this study is to determine the threshold infection levels of WSSV in different weight ranges of *P. vannamei* using qPCR and to know if it is weight dependent. The established threshold infection levels will enable the farmers to strictly monitor the health status of the cultured shrimps, and it will also serve as a reference for the ideal viral load to maintain in the farm to avoid disease outbreaks.

Materials and methods

Screening and maintenance of experimental animals

Experimental animals (*P. vannamei*) were obtained from a shrimp grow-out farm in Zarraga, Iloilo. The weight ranges needed for the infection experiments were 3–5 g, 7–8 g, 15–18 g, and 22–25 g. Before the procurement and stocking, the experimental animals were screened for WSSV and AHPND using conventional PCR. If the results were negative, the experimental animals were stocked in 250 L fiberglass tanks and maintained in a flow-through seawater system at salinity of 29–32 ppt, water temperature of 25–30 °C, and constant supply of aeration and UV-sterilized seawater at the Infection Building, Wet Laboratory Complex, SEAFDEC/AQD. Commercial shrimp feed was given twice a day (9:00 AM and 3:00 PM) to the shrimp stocks with feeding rate of 3 % body weight.

Inoculum preparation and tissue passage

The inoculum was prepared by homogenizing WSSV one-step positive shrimp tissues (de la Peña *et al*., 2015). Approximately 0.75 g of muscle tissue was homogenized with 6.75 ml of phosphate-buffered saline (PBS) and centrifuged at 9,000 rpm for 10 min at 4 °C. The supernatant was then filtered with a 0.45 µm pore size syringe filter. The supernatant containing the virus was diluted with the ratio of 1:10 parts (filtrate: PBS; 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}). The dilution used for the tissue passage was 10^{-4}.

Ten aquaria were filled with 10 L of UV-sterilized seawater with salinity of 30 ppt, supplied with constant aeration, and the water temperature was maintained at 25 °C. In each aquarium, ten shrimps (ABW 18 g) were stocked and acclimatized for 8 hours (h). The positive control groups were intramuscularly injected with 100 µl of the viral inoculum while 100 µl of PBS was given to the negative control. Mortalities were monitored and recorded every 6 h. The dead shrimps were collected, dissected into different sections (head, body, and tail), placed and labeled in an individual resealable plastic bag, and stored at -80 °C biofreezer. Two tissue passages were conducted to increase the virulence of the viral isolate and the volume of infected tissues. Conventional PCR analysis was conducted to confirm that the shrimps were successfully infected with WSSV. The shrimp samples that were 1-step PCR positive for WSSV were used as a source of viral inoculum for the following artificial infection experiments.

DNA extraction

Total genomic DNA was extracted from the gill tissue of the infected shrimps using DNAzol Reagent (MRC, USA) and following the manufacturer's protocol. Briefly, approximately 50 mg of gill tissue were homogenized in 1 ml DNAzol, followed by centrifugation for 10 min at 14,800 x g at 4 °C and transfer of the supernatant to a new tube. DNA was precipitated by the addition of 0.5 ml of 100 % ethanol. Pelleted DNA was washed twice with 1 ml of 75 % ethanol by centrifugation and air-dried for a few seconds. The dried DNA pellets were suspended in 100 µl of 8 mM NaOH, incubated at 45 °C for 15 min, after which 10 µl of TE buffer was added for storage at -20 °C. The concentration and purity of the extracted DNA were measured using a nano-spectrophotometer (IMPLEN, Germany).

Detection of WSSV using conventional PCR

The infected shrimp samples were submitted to l-step and nested PCR tests using the WSSV -specific primer pairs designed by Kimura *et al.* (1996). PCR reactions were carried out in a 25 µl reaction mixture. Amplification was performed in a programmable thermal cycler (Eppendorf, Germany) with the following cycle parameters: the initial heating at 72 °C for 10 min and 95 °C for 6 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 1 min with a final extension at 72 °C for 5 min before holding at 4 °C until ready for electrophoresis. For the nested PCR step, 1.0 µl of the post-1-step PCR was used as the template for PCR amplification using the primer pair P3-P4 with the protocol described above.

The PCR products were separated in 2 % agarose gel, stained with Gel Red Nucleic Acid Stain (Biotium, USA), and visualized using the DigiDoc-It® Imaging System (Analytik Jena, USA). The one-step and nested primer pairs amplified products were 982 bp and 570 bp, respectively.

Determination of viral load using qPCR

Rotor-Gene RG3000 (Corbett Research, Australia) real-time PCR machine and fluorescent dye KAPA SYBR® FAST qPCR Master Mix Kits (KAPA Biosystems, USA) were used for the real-time PCR analysis of the WSSV viral load. The amplification profile was performed and programmed

in the Rotor-Gene RG3000 with the following cycle parameters: the initial heating at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 1 min and extension at 72 °C for 1 min with a final extension at 72 °C for 5 min. Real-time PCR was conducted in a 13 µl reaction using 1 µl of the original viral inoculum (unfiltered, undiluted preparation), 6.25 µl of SYBR Green Master Mix, 0.25 µl of each P1-P2 primer (Kimura *et al.* 1996; 10 mM final concentration), and 4.75 µl of ultrapure distilled water (Invitrogen, USA). In each of the 36-wells real-time PCR run, a dilution series of the plasmid standard of WSSV was run along with the unknown samples and no template controls (NTC).

Determination of LD_{50}

Preliminary infection experiments using exponential serial dilutions were performed to determine the range of inoculum for LD_{50}. The viral load of the prepared inoculum was determined using qPCR before the preliminary infection experiment. Six dilutions (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10}) of the viral inoculum were prepared from WSSV infected shrimp tissue.

Individual LD_{50} was conducted to four weight ranges. The experimental set-ups consist of 10 aquaria filled with 10 L of UV-sterilized seawater with salinity of 30 ppt, supplied with constant aeration, and water temperature maintained at 25 °C. In each aquarium, ten shrimps were stocked and acclimatized for 8 h before the injection of the viral inoculum. The experimental groups were injected intramuscularly with 100 µl of the viral inoculum, and the negative control groups received 100 µl of PBS. The shrimps were monitored and observed for mortalities every 6 h. The dead shrimps were collected, dissected into different sections (head, body, and tail), and stored at -80 °C. The rearing water was changed every day by 50 % through siphoning.

Time-course experiment

Time-course experiments were conducted on all four different weight ranges after the appropriate viral dilution was determined during the LD_{50}. The experimental set-ups consist of 10 aquaria filled with 10 L of UV-sterilized seawater, salinity of 30 ppt, supplied with constant aeration, and water temperature maintained at 25 °C. In each aquarium, ten shrimps were stocked and acclimatized for 8 h before injection of the viral inoculum. The experimental groups were intramuscularly injected with 100 µl of the 10^{-6} dilution of the viral inoculum, and the negative control received only 100 µl of PBS. The proper time interval of sampling was followed in order to monitor the increase of the viral load. Every sampling time, 2 shrimps were collected, dissected into different sections (head, body, and tail), and stored at -80 °C biofreezer prior to the qPCR test. The rearing water was changed every day by 50 % through siphoning.

Results

Screening of experimental animals

Several batches of experimental animals were all nested PCR negative for WSSV and AHPND, as shown in **Figures 1** and **2**.

Tissue passage

To increase the virulence of WSSV, at least two tissue passages were conducted. The first batch of 35 shrimps (ABW 18 g) were all one-step PCR positive. For the second

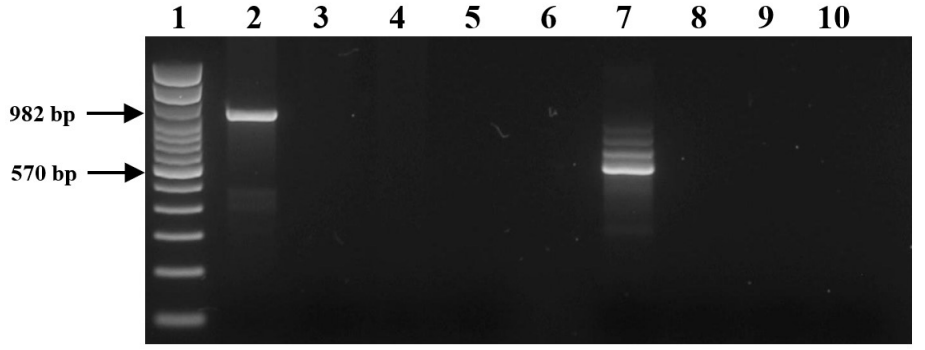

Figure 1. Agarose gel electrophoresis of the products from WSSV 1-step and nested PCR amplifications using specific primers designed by Kimura et al. (1996). Lanes (1) 100 bp DNA marker, (2) 1-step positive control, (3-4) experimental shrimps, (5) negative control, (6) empty, (7) nested positive control, (8-9) experimental shrimps, and (10) negative control

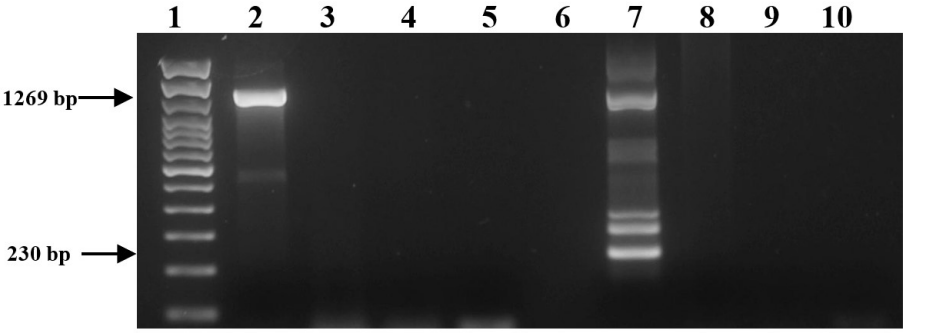

Figure 2. Agarose gel electrophoresis of the products from AHPND 1-step and nested PCR amplifications using specific primers designed by Dangtip et al. (2015). Lanes (1) 100 bp DNA marker, (2) 1-step positive control, (3-4) experimental shrimps, (5) negative control, (6) empty, (7) nested positive control, (8-9) experimental shrimps, and (10) negative control

batch of 40 shrimps (ABW 18 g), 30 out of 40 were found to be one-step PCR positive. Viral loads of infected shrimp from the tissue passages were determined using qPCR. The viral loads of 1-step PCR positive shrimps ranged from 2.7 x 10^9 to 5.1 x 10^9 WSSV DNA copies/g, while the nested PCR positive shrimps ranged from 6.4 x 10^6 to 8.9 x 10^6 WSSV DNA copies/g.

Establishment of the standard curve for qPCR

The optimization and establishment of the qPCR protocol were earlier conducted under the Government of Japan-Trust Fund 5 (GOJ-TF 5) Project. WSSV-infected muscle tissues and WSSV-purified plasmid were used in the establishment of the standard curve. The plasmid concentration was estimated to be 4.40 x 10^{10} WSSV DNA copies/µl. Serial 4-fold dilutions of the plasmid were used for the standard curve to quantify WSSV genomic DNA. As shown in **Figure 3**, the standard curve has an R^2 equal to 0.98466. The real-time PCR protocol has a detection limit of 33 DNA copies/µl (unpublished results).

Determination of LD_{50}

Six dilutions (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10}) of the viral inoculum were prepared and used for the preliminary infection experiments. Summary of the viral inoculum dilution and number of days for the LD_{50} were shown in **Table 1**. For weight ranges 3–5 g and 7–8 g, the LD_{50} was determined at the dilution of 10^{-6} and was achieved after 216 and 240 hours post-infection (hpi), respectively. While for weight ranges 15–18 g and 22–25 g, the LD_{50} was at the dilution of 10^{-5} and was achieved after 216 and 240 hpi, respectively. The viral load of the 10^{-6} dilution of inoculum was 7.7 x 10^5 DNA copies/g, while for 10^{-5} was 6.1 x 10^6 DNA copies/g.

Determination of the threshold infection levels

Summary of the average viral loads of each sampling point for all the time-course infection experiments were presented in **Table 4**. The mortalities of weight range 3–5 g have started at 195 hpi with a viral load ranging from 1.6 x 10^9 to 3.3 x 10^9 WSSV DNA copies/g. While the range of the viral load of the survivors were from 3.3 x 10^6 to 4.1 x 10^6 WSSV DNA copies/g after 231 hpi. The mortalities of weight range 7–8 g have started at 231 hpi with a viral load ranging from 3.7 x 10^9 to 5.1 x 10^9 WSSV DNA copies/g. While the range of the viral load of the survivors were from 6.3 x 10^6 to 8.9 x 10^6 WSSV DNA copies/g after 264 hpi. The mortalities of weight range 15-18 g have started at 162 hpi with a viral load ranging from 1.2 x 10^9 to 4.6 x 10^9 WSSV DNA copies/g. While the range of the viral load of the survivors were from 6.8 x 10^6 to 8.8 x 10^6 WSSV DNA copies/g after 219 hpi. The mortalities of weight range 22–25 g have started at 237 hpi with a viral load ranging from 1.4 x 10^9 to 5.1 x 10^9 WSSV DNA copies/g. While the range of the viral load of the survivors were from 5.1 x 10^6 to 9.3 x 10^6 WSSV DNA copies/g after 255 hpi.

The mortalities, survival, and manifestation of clinical signs were carefully correlated in determining the threshold level of WSSV in shrimp. As shown in **Table 4**, there were late onsets on the increase of viral loads observed for weight ranges 3–5 g and 7–8 g, which took about 183–219 hpi to increase from 10^6 to 10^7 WSSV DNA copies/g. On the contrary, for weight ranges, 15–18 g and 22–25 g, early onsets of viral load increase were observed around 24–48 hpi to increase from 10^6 to 10^7 WSSV DNA copies/g. As the viral load reached 109 WSSV DNA copies/g, clinical signs and mortalities were observed.

Discussion

From the first emergence of WSSV until the early 2000s, the approximate economic loss of Asian countries was suggested to reach US$ 1 Billion per annum (Briggs *et al.*,

Table 1. Viral inoculum dilutions and time to reach LD_{50} in 4 weight ranges

Weight ranges	LD_{50} Viral dilution	Hours post-infection (hpi)
3-5	10^{-6}	216 hpi
7-8	10^{-6}	240 hpi
15-18	10^{-5}	216 hpi
22-25	10^{-5}	240 hpi

Table 2. Viral loads of 4 weight ranges during the time-course infection experiments that mortalities have started

Weight ranges	LD_{50} Viral dilution	Average
3-5	1.60×10^9 - 3.30×10^9	2.58×10^9
7-8	3.70×10^9 - 5.10×10^9	4.48×10^9
15-18	1.20×10^9 - 4.60×10^9	3.36×10^9
22-25	1.40×10^9 - 5.10×10^9	3.81×10^9

Table 3. Viral loads of the survivors of 4 weight ranges during the time-course infection experiments

Weight ranges	LD_{50} Viral dilution	Average
3-5	3.30×10^6 - 4.10×10^6	23.86×10^6
7-8	6.30×10^6 - 8.90×10^6	7.62×10^6
15-18	6.80×10^6 - 8.80×10^6	7.90×10^6
22-25	5.10×10^6 - 9.30×10^6	7.48×10^6

Table 4. Summary of the viral loads of the samples of 4 weight ranges during the time course infection experiments

3-5 g		7-8 g		15-18 g		22-25g	
hpi	WSSV DNA copies/g	hpi	WSSV DNA copies/g	hpi	WSSV DNA copies/g	hpi	WSSV DNA copies/g
0	3.79×10^6	0	3.71×10^6	0	3.78×10^6	0	3.71×10^6
24	4.03×10^6	24	4.36×10^6	24	5.64×10^7	24	6.75×10^6
48	3.93×10^6	48	3.90×10^6	48	2.34×10^7	48	1.05×10^7
72	3.52×10^6	72	5.00×10^6	72	8.04×10^7	72	1.77×10^7
96	3.00×10^6	96	4.70×10^6	96	2.31×10^8	96	2.85×10^7
120	3.74×10^6	120	6.04×10^6	120	4.72×10^8	120	4.07×10^7
132	4.10×10^6	144	5.90×10^6	132	3.82×10^8	144	3.90×10^7
144	4.24×10^6	156	6.50×10^6	144	7.88×10^8	156	7.40×10^7
156	3.93×10^6	168	7.00×10^6	156	9.89×10^8	168	8.47×10^7
162	4.00×10^6	180	6.90×10^6	162	1.28×10^9	180	7.00×10^7
168	3.23×10^6	192	8.15×10^6	168	2.01×10^9	192	9.98×10^7
174	4.15×10^6	198	7.74×10^6	174	1.90×10^9	198	8.97×10^7
180	3.50×10^6	204	9.01×10^6	180	3.92×10^9	204	8.96×10^7
183	1.04×10^7	210	8.80×10^6	183	2.89×10^9	210	9.91×10^7

3-5 g		7-8 g		15-18 g		22-25g	
hpi	WSSV DNA copies/g	hpi	WSSV DNA copies/g	hpi	WSSV DNA copies/g	hpi	WSSV DNA copies/g
186	5.05×10^{7}	213	9.99×10^{6}	186	2.10×10^{9}	213	8.45×10^{7}
189	2.01×10^{8}	216	8.48×10^{6}	189	3.60×10^{9}	216	7.34×10^{7}
192	7.30×10^{8}	219	7.86×10^{7}	192	4.00×10^{9}	219	9.86×10^{7}
195	1.96×10^{9}	222	1.46×10^{8}	195	4.92×10^{9}	222	9.85×10^{7}
198	2.01×10^{9}	225	1.94×10^{8}	198	3.83×10^{9}	225	7.85×10^{7}
201	3.02×10^{9}	228	9.47×10^{8}	201	2.90×10^{9}	228	9.56×10^{7}
204	1.98×10^{9}	231	4.61×10^{9}	204	4.10×10^{9}	231	6.58×10^{7}
207	2.79×10^{9}	234	3.71×10^{9}	207	4.59×10^{9}	234	9.41×10^{7}
210	1.68×10^{9}	237	4.04×10^{9}	210	3.79×10^{9}	237	1.40×10^{9}
213	3.03×10^{9}	240	3.99×10^{9}	213	4.19×10^{9}	240	2.97×10^{9}
216	3.00×10^{9}	243	4.57×10^{9}	216	3.75×10^{9}	243	3.73×10^{9}
219	2.94×10^{9}	246	4.54×10^{9}	219	6.84×10^{6}	246	5.15×10^{9}
225	3.37×10^{9}	249	4.19×10^{9}	225	$8,.82 \times 10^{6}$	249	5.00×10^{9}
231	3.49×10^{6}	252	5.05×10^{9}	231	7.45×10^{6}	252	4.60×10^{9}
237	3.93×10^{6}	255	4.93×10^{9}	237	8.43×10^{6}	255	5.13×10^{6}
243	4.14×10^{6}	258	4.57×10^{9}	243	7.42×10^{6}	258	5.89×10^{6}
243	4.00×10^{6}	261	5.12×10^{9}	243	7.20×10^{6}	261	7.94×10^{6}
255	3.83×10^{6}	264	7.04×10^{6}	255	7.42×10^{6}	264	7.30×10^{6}
279	3.59×10^{6}	267	8.39×10^{6}	279	8.01×10^{6}	267	6.73×10^{6}
303	4.22×10^{6}	270	6.31×10^{6}	303	8.74×10^{6}	270	5.76×10^{6}
327	3.65×10^{6}	276	7.80×10^{6}	327	8.64×10^{6}	276	9.36×10^{6}
		282	6.34×10^{6}			282	7.04×10^{6}
		288	8.91×10^{6}			288	8.50×10^{6}
		294	8.12×10^{6}			294	7.59×10^{6}
		306	7.84×10^{6}			306	9.31×10^{6}
		318	7.77×10^{6}			318	9.15×10^{6}

2005). This massive economic loss can be attributed to high mortality rates that can reach up to 100 % within 10 days (Flegel, 1997). Even with the advancement of shrimp aquaculture systems, like the semi-closed, use of pond liners, shrimp "toilet", and biofloc, disease outbreaks caused by WSSV is still considered as the number one threat in the sustainability of the industry worldwide.

In this study, the LD_{50} of the different weight ranges (3–5 g, 7–8 g, 15–18 g, and 22–25 g) of shrimps were achieved at viral

dilution of 10^{-6} and 10^{-5} after 216–240 hpi. The viral loads of the inoculum have a range of 10^5–10^6 WSSV DNA copies/g. These viral loads were parallel to the results of Durand and Lightner (2002), Meng *et al.* (2010), and Jeswin *et al.* (2013) that the presence of 10^5 to 10^7 WSSV DNA copies/g is sufficient to infect shrimps through immersion or intramuscular injection. The LD_{50} results were used to determine the appropriate viral inoculum to be injected and the sampling frequencies of the time-course infection experiments.

The average viral loads present in the samples when the mortalities have started in all the time-course infection experiments were 2.58 x 10^9, 4.48 x 10^9, 3.36 x 10^9, and 3.81 x 10^9 WSSV DNA copies/g, while for the surviving shrimps were 3.86 x 10^6, 7.62 x 10^6, 7.90 x 10 10^6, and 7.48 x 106 WSSV DNA copies/g, for weight ranges 3–5 g, 7–8 g, 15–18 g, and 22–25 g, respectively. Tang and Lightner (2000) were able to classify the infection level of WSSV in juvenile shrimps (1 g) based on the relationship between the viral load of the samples and the severity of the infection. According to their classification, the average viral loads of the samples from all the time-course infection experiments when the mortalities occurred were under moderate to severe (G3, 2 x 10^9 WSSV DNA copies/g) classification, while the survivors were classified as mild (G1, 2 x 10^5 WSSV copies/g). Based on their observations, samples with infection level G3 became moribund after 35–60 hpi. Our observations for weight ranges 3–5 g, 7–8 g, 15–18 g, and 22–25 g the mortalities started at 195 hpi, 231 hpi, 162 hpi, and 237 hpi, respectively. Furthermore, the viral loads of other penaeid shrimps that were subjected to artificial infection experiments which resulted in mortalities were quantified: in artificially infected juvenile *P. vannamei* (7.50 x 10^8 to 2.5 x 10^9 copies/µg of total DNA) and juvenile *Feneropenaeus chinensis* (5.72 x 10^5 to 9.6 x 10^5 copies/ng of DNA) (Durand and Lightner, 2002; Sun *et al.*, 2012).

According to Sun *et al.* (2012), the turning point of chronic infection to acute infection in juvenile *F. chinensis* (4–8 g) was suggested from 27 to 30 hpi when the viral load increased from 10^3 to 10^4 copies/ng of DNA. Moreover, the substantial increase of the viral load to 10^5 copies/ng of DNA explained the symptoms of skin color changes, reduced food consumption, and gathering at the water surface due to dyspnea, resulting in mortality. In our time-course experiments, we observed that the viral loads of the lighter weight ranges 3–5 g and 7–8 g increased by one exponential order (from 10^6 to 10^7 WSSV DNA copies/g) after 180 to 216 hpi, while the heavier weight ranges 15–18 g and 22–25 g were earlier after 24 to 48 hpi. However, no clinical signs and mortalities were observed in this viral load range (10^6 to 10^7 WSSV DNA copies/g). It was only when the viral load reached up to 10^9 WSSV DNA copies/g that clinical signs and mortalities were observed.

Viral loads of WSSV positive shrimp submitted to Fish Health Section Diagnostic Laboratory, SEAFDEC/AQD were also determined. This was done to compare the viral loads of artificially and naturally infected shrimp samples. The WSSV positive samples were divided into two groups, 1-step and nested. The viral loads of naturally infected 1-step and nested PCR positive samples ranged from 3.2 x 10^9 to 5.1 x 10^9 WSSV DNA copies/g and 7.4 x 10^3 to 1.2 x 10^t WSSV DNA copies/g, respectively. Mendoza-Cano and Sanchez-Paz (2013) were able to determine the viral load of WSSV in wild samples of marine crustaceans: *Macrobrachium rosenbergii* (3.4 x 10^7 DNA copies/ µl), *Penaeus vannamei* (1.28 x 10^6 DNA copies/ µl), *P. stylirostris* (2.13 x 10^4

DNA copies/µl), *Callinectes bellicosus* (5.98 x 10^3 DNA copies/µl) and *Calanus pacificus californicus* (6.1 x 10^5 DNA copies/µl). Also, our results conformed to the quantification of naturally infected cultured penaeid shrimps (2.1 x 10^8 to 2.64 x 10^{14} copies/g of shrimp tissue) (Siddique *et al*., 2018).

The time-course infection experiment was designed based on the results of LD_{50} in different weight ranges of shrimp. The viral inoculum used was 10^{-6} with a viral load of 10^5 WSSV DNA copies/g. Also, we quantified and correlated the viral loads of the naturally and artificially infected shrimps. The viral load of naturally infected nested PCR positive samples (10^3–10^5 DNA copies/g) was less than compared to the viral inoculum used in the LD50, while the 1-step PCR positive samples (10^9 WSSV DNA copies/g) were more than compared to the established threshold infection level that resulted in mortalities in artificially infected shrimps. Based on the viral loads of artificially and naturally infected shrimps, we elucidated that the threshold infection levels of WSSV in *P. vannamei* was from 10^7 to 10^8 WSSV DNA copies/g. In this viral load range, there were no observable clinical signs and mortalities. Also, we have observed that in all weight ranges, the threshold infection levels are the same. Therefore, it is not weight dependent.

To date, there is no available data on the threshold infection levels of WSSV in penaeid shrimps. Hence, in this study, we established the threshold infection levels of WSSV in different weight ranges of *P. vannamei* from 10^7 to 10^8 WSSV DNA copies/g. These established threshold infection levels can be used as a reference by shrimp farmers and laboratory analysts to facilitate stringent and proactive health monitoring of the shrimp stocks on the farm. Also, it will give the farmers adequate time to implement appropriate interventions before the viral load reaches the threshold infection levels that will result in WSSV outbreak.

Acknowledgment

We are very grateful to the Government of Japan-Trust Fund (study code: FH-07-F2015T) and SEAFDEC/AQD for funding this work. We thank Mr. Ivan Yaptangco of IJY Shrimp Farm for providing the experimental animals and the staff of Fish Health Section Diagnostic Services for the technical support.

References

Briggs, M., S. Funge-Smith, R.P. Subasinghe and M. Phillips. 2005. Introductions and movement of two penaeid shrimp species in Asia and the Pacific. FAO Fisheries Technical Paper. No. 476. FAO, Rome. 78 pp

Cuéllar-Anjel, J., White-Noble, B., Schofield, P., Chamorro, R., & Lightner, D. V. 2012. Report of significant WSSV-resistance in the Pacific white shrimp, *Litopenaeus vannamei*, from a Panamanian breeding program. Aquaculture, 368–369, 36–39. https://doi.org/10.1016/j.aquaculture.2012.08.048

de la Peña, D., Lavilla-Pitogo, C., Villar, C., Paner, M., Sombito, C., & Capulos, G. 2007. Prevalence of white spot syndrome virus (WSSV) in wild shrimp *Penaeus monodon* in the Philippines. Diseases of Aquatic Organisms, 77, 175–179. https://doi.org/10.3354/dao01834

de la Peña, L., Cabillon, N., Catedral, D., Amar, E., Usero, R., Monotilla, W., Calpe, A., Fernandez, D., & Saloma, C. 2015. Acute hepatopancreatic necrosis disease (AHPND) outbreaks in *Penaeus vannamei* and *P. monodon* cultured in the Philippines. Diseases of Aquatic Organisms, 116(3), 251–254. https://doi.org/10.3354/dao02919

Durand, S. V., & Lightner, D. V. 2002. Quantitative real time PCR for the measurement of white spot syndrome virus in shrimp. Journal of Fish Diseases, 25(7), 381–389. https://doi.org/10.1046/j.1365-2761.2002.00367.x

Fish Vet Group Asia Limited, Muang Chonburi, Chonburi, Thailand, & Shinn, A. P. 2018. Asian Shrimp Production and the Economic Costs of Disease. Asian Fisheries Science, 31S. https://doi.org/10.33997/j.afs.2018.31.S1.003

Flegel, T. W. 1997. Special topic review: Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. 13, 10.

Hoa, T. T. T., Zwart, M. P., Phuong, N. T., Vlak, J. M., & de Jong, M. C. M. 2011. Transmission of white spot syndrome virus in improved-extensive and semi-intensive shrimp production systems: A molecular epidemiology study. Aquaculture, 313(1–4), 7–14. https://doi.org/10.1016/j.aquaculture.2011.01.013

Huang, Y.-C., Yin, Z.-X., Ai, H.-S., Huang, X.-D., Li, S.-D., Weng, S.-P., & He, J.-G. 2011. Characterization of WSSV resistance in selected families of *Litopenaeus vannamei*. Aquaculture, 311(1–4), 54–60. https://doi.org/10.1016/j.aquaculture.2010.11.032

International Office of Epizootics. (2019). Aquatic animal health code.

Jeswin, J., Anju, A., Thomas, P. C., Paulton, M. P., & Vijayan, K. K. 2015. Analysis of viral load between different tissues and rate of progression of white spot syndrome virus (WSSV) in *Penaeus monodon*. Aquaculture Research, 46(8), 2003–2012. https://doi.org/10.1111/are.12357

Kasornchandra, J., Boonyaratpalin, B., Khongpradit, R., & Akpanithanpong, U. 1995. Mass mortality caused by systemic bacilliform virus in cultured Penaeid shrimp, *Penaeus monodon*. *Thailand. Asian Shrimp News*, 5(2), 2-3.

Kimura T., Yamano K., Nakano H., Momoyama K., Hiraoka M., & Inouye K. 1996. Detection of Penaeid Rod-shaped DNA Virus(PRDV) by PCR. Fish Pathology, 31(2), 93–98. https://doi.org/10.3147/jsfp.31.93

Lotz, J. M. 1997. Special topic review: Viruses, biosecurity and specific pathogen-free stocks in shrimp aquaculture. 13, 9.

Lightner, D. V. 1996. A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp.

Magbanua, F., Natividad, K., Migo, V., Alfafara, C., de la Peña, F., Miranda, R., Albaladejo, J., Nadala, E., Loh, P., & Mahilum-Tapay, L. 2000. White spot syndrome virus (WSSV) in cultured *Penaeus monodon* in the Philippines. Diseases of Aquatic Organisms, 42, 77–82. https://doi.org/10.3354/dao042077

Mendoza-Cano, F., & Sánchez-Paz, A. 2013. Development and validation of a quantitative real-time polymerase chain assay for universal detection of the White Spot Syndrome Virus in marine crustaceans. Virology Journal, 10(1), 186. https://doi.org/10.1186/1743-422X-10-186

Meng, X.-H., Jang, I. K., Seo, H.-C., & Cho, Y.-R. 2010. A TaqMan real-time PCR assay for survey of white spot syndrome virus (WSSV) infections in *Litopenaeus vannamei* postlarvae and shrimp of farms in different grow-out seasons. Aquaculture, 310(1–2), 32–37. https://doi.org/10.1016/j.aquaculture.2010.10.010

Siddique, M. A., Haque, Md. I.-M., Sanyal, S. K., Hossain, A., Nandi, S. P., Alam, A. S. M. R. U., Sultana, M., Hasan, M., & Hossain, M. A. 2018. Circulatory white spot syndrome virus in South-West region of Bangladesh from 2014 to 2017: Molecular characterization and genetic variation. AMB Express, 8(1), 25. https://doi.org/10.1186/s13568-018-0553-z

Sun, Y., Li, F., & Xiang, J. 2013. Analysis on the dynamic changes of the amount of WSSV in Chinese shrimp *Fenneropenaeus chinensis* during infection. Aquaculture, 376–379, 124–132. https://doi.org/10.1016/j.aquaculture.2012.11.014

Tang, K. F. J., & Lightner, D. V. 2000. Quantification of white spot syndrome virus DNA through a competitive polymerase chain reaction. Aquaculture, 189(1–2), 11–21. https://doi.org/10.1016/S0044-8486(00)00367-7