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White spot syndrome virus quantification in blue crab *Portunus trituberculatus* hatchery-produced larvae and wild populations by TaqMan real-time PCR, with an emphasis on the relationship between viral infection and crab health

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ABSTRACT

The blue crab, *Portunus trituberculatus*, is one of the most important fishery resources in the Yellow Sea of Korea, but the wild stock of this species has been reduced due to over-fishing and the destruction of the natural habitat in this area. Hatchery-produced seeds of blue crab have been released into the sea to enhance stock, but information on viral infection in the larvae as well as wild crabs of this species is very limited. In the present study, TaqMan real-time PCR was applied to quantify white spot syndrome virus (WSSV) in hatchery-produced larvae and wild populations of *P. trituberculatus* in South Korea. Out of 140 *P. trituberculatus* zoea from seven commercial hatcheries, 96.4% were WSSV-positive. The mean WSSV copies were 6.0 per ng DNA, or 3216.0 per larva. In 222 adult crabs from four wild populations captured in different seasons, the WSSV-positive rate was 79.3%, and the WSSV load was 5.2 copies per ng DNA, or 2116.5 copies per mg tissue. Both the WSSV-positive rate and the load of the winter population were significantly lower than those of the other three populations. Statistical analysis showed no significant correlations between WSSV infection loads and growth (CL, CW, and BW). The results suggest that low viral load of WSSV may not affect the growth of *P. trituberculatus* in a wild environment.

1. Introduction

White spot syndrome virus (WSSV) has been the most serious epizootic to affect cultured shrimp since WSSV was found in East Asia in 1992/3 (Chen, 1995; Chou et al., 1995; Lightner, 1996; Flegel, 1997). In South Korea, WSSV has caused great economic loss to shrimp farmers since then and, in particular, accounted for more than 30% of the farms experiencing total or partial loss of shrimp before harvest in 2004 (Kim et al., 1997; Heo et al., 2000; Jang et al., 2007a,b).

WSSV has a wide host range that includes species of not only penaeid and non-penaeid shrimp but also other crustaceans such as marine crabs (Lo et al., 1996; Chang et al., 1998, 2001; Kanchanaphum et al., 1998; Wang et al., 1998; Corbel et al., 2001; Sahul Hameed et al., 2003), freshwater crabs (Sahul Hameed et al., 2001) and lobsters (Chang et al., 1998; Rajendran et al., 1999). Previous studies showed that WSSV also infects many species of wild crabs, including some portunids, *Charybdi feriatus*, *Portunus pelagicus* and *P. sanguinolentus* (Lo et al., 1996); *Charybdis cruciata* and *Matuta planipes* (Otta et al., 1999); the American blue crab, *Callinectes sapidus* (Chang et al., 2001); *Charybdis annulata*, *Macrophthalmus sulcatus*, *Gelasimus marionis nitidus* and *Metopograpsus messor* (Hossain et al., 2001); and the mud crab, *Scylla serrata* larvae (Chen et al., 2000). Based on the above studies and artificial infection trials, it is suggested that the crab's susceptibility to WSSV is species-specific, as in shrimp and lobster (Rajendran et al., 1999; Corbel et al., 2001; Sahul Hameed et al., 2001). For example, Sahul Hameed et al. (2003), through the artificial infection of marine crabs with WSSV, found that only four among 20 species act as latent carriers without mortality. The blue crab, *P. trituberculatus*, is also susceptible to WSSV and exhibits mortality due to WSSV in cultured and artificially infected conditions. Moribund and dead blue crabs severely infected by WSSV are always characterized by typical histopathological changes in the gill, hepatopancreas and heart (Zhan et al., 2000; Wang et al., 2006).

P. trituberculatus has a wide geographic distribution ranging from Korea, Japan, China, through Southeast Asia to the Indian Ocean (Sakai, 1939; Kim, 1973; Dai et al., 1986). This crab is one of the most important fishery resources in the Yellow Sea of Korea, but the natural resources of this species has been reduced due to over-fishing and the destruction of the natural habitat including spawning grounds in this area. The dramatic decrease in fishery capture of this species in Korea (from 18,195 MT in 2002 to 2,295 MT in 2004) reflects the seriousness of the depletion of wild stock (Yeon et al., 2008). To enhance the wild stock of *P. trituberculatus*, millions of hatchery-produced juveniles have been released into the Yellow Sea off the coast of Korea every year since the 1980s (MOMAF, 2002–2006). Recently, the Korean

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Table 1
Sample information and WSSV load of *P. trituberculatus* larvae collected from seven hatcheries in 2007.

Hatchery (larvae stage)	Sampling date	Location	No. of specimens	Copies/ng DNA	Copies/ind.	Prevalence of positive (%)
H_1 (Z_2)	Jun. 7	Seosan	20	6.5 ± 4.1	2016.4 ± 1260.4	100
H_2 (Z_2)	Jun. 7	Taean	20	7.5 ± 8.2	2262.4 ± 2491.0	95
H_3 (Z_2)	Jun. 8	Taean	20	3.9 ± 3.7	2258.9 ± 1890.9	90
H_4 (Z_2)	Jun. 8	Taean	20	2.0 ± 1.6	957.0 ± 784.4	100
H_5 (Z_2)	Jun. 8	Taean	20	2.5 ± 1.9	1478.3 ± 1106.1	90
H_6 (Z_2)	Jun. 15	Boryeong	20	13.0 ± 33.2	10789.1 ± 27499.5	100
H_7 (Z_3)	Jun. 26	Haenam	20	6.9 ± 8.1	2976.1 ± 3507.7	100
	Total		140	6.0 ± 13.6	3216.0 ± 10809.2	96.4

government began using the nested PCR method to strictly screen crab larvae for diseases including WSSV before release in order to enhance the health of the wild stock. Viral infection of *P. trituberculatus* larvae is closely related to natural stock and vice versa because in Korea the larval production entirely depends on wild brooders. Considering the commercial importance in fisheries as well as the health of the ecosystem, a quantitative study of WSSV infection in wild adults and hatchery-produced seeds is very important, but this information regarding the Yellow Sea has not been available to date.

The present study assayed WSSV infection in hatchery-produced *P. trituberculatus* larvae and wild populations in the Yellow Sea of South Korea. A TaqMan real-time PCR method was applied to evaluate the quantitative assay of WSSV infection. A statistical analysis was also conducted to estimate the relationship between viral infections and the health of the crabs.

2. Materials and methods

2.1. Collection of experimental animals

P. trituberculatus zoea were collected from 7 June 2007 through 26 June 2007 from seven commercial hatcheries located in the Yellow Sea off the coast of South Korea (Table 1). In the same year, four adult wild populations were caught using fishing boats from Jindo-Is. (P_J, 29 inds.) in April when collection of the wild crabs is available in Korea, Taean (P_T, 20 inds.) in June, Anmyeondo-Is. (P_A, 120 inds.) in August and Daecheon (P_D, 53 inds.) in December (Table 2). The later three locations are within 50 km, except Jindo-Is. is apart about 200 km from the three locations. All specimens that were alive were transported to the West Sea Mariculture Research Center of the National Fisheries Research & Development Institute (NFRDI), Taean, South Korea and individually measured: the larval stage and body weight for zoea and the carapace length (CL), carapace width (CW), and body weight (BW) for adult crabs. A pereiopod of each crab and the whole body of the larvae were preserved in 70% alcohol for genomic DNA extraction. All adult crabs and 20 larvae of each hatchery were assayed for WSSV quantification.

2.2. WSSV quantification method

The method of DNA extraction, preparation of standard plasmid and amplification of real-time PCR as well as the sequence of primers and probe used for WSSV detection were performed as described by Jang et al. (2009). By Rotor Gene 6000 (Corbett Research Inc., Australia), all samples were run in triplicate with three non-template controls (NTC) as negative controls for each new run. The viral copy number was normalized on an ng genomic DNA basis, on an mg tissue basis (for adult crabs only) and on an individual basis (for larvae only) based on precise amount of tissue or larvae used for DNA extraction.

2.3. Data analysis

The software package SPSS 7.5 (SPSS Inc., Chicago, IL, USA; available at http://www.spss.com) was applied to conduct statistical analysis. To determine the relationship between the WSSV load and the crab's health, the correlation of the WSSV load and CL, CW, and BW of populations P_A and P_D was analyzed; in addition, the biomass (CL, CW, and BW) of WSSV positive- and negative-individuals were compared. The difference between viral loads in female and male crabs was also analyzed.

3. Results

3.1. Standard curve

Strong linear correlations ($R^2 = 0.99832$) were obtained between the threshold cycles (Ct) and the target plasmid standard ranging from 5.9×10^6 to 5.9×10^0 WSSV copies in PCR with high reaction efficiency ($E = 0.97$) and proper slope ($M = -3.408$). With an optimal PCR mixture, 10^9 to 2 WSSV copies with the genomic DNA of the specimen were detected in one reaction, which indicated the large dynamic range and high sensitivity of the assay.

3.2. WSSV infection of P. trituberculatus zoea

A total of 140 *P. trituberculatus* larvae in stage Z_2–Z_3 from seven commercial hatcheries were assayed, and 96.4% tested positive for WSSV (Table 1). Among the seven hatcheries, four (H_1, H_4, H_6, and H_7) tested 100% positive for WSSV. The prevalence of WSSV infection in H_2, H_3, and H_5 was 95%, 90%, and 90%, respectively. The mean WSSV infection of the seven hatcheries was 6.0 copies ng^{-1} DNA, or 3216.0 copies per individual. The range of WSSV infection was 2.0–13.0 copies ng^{-1} DNA or 957.0–10789.1 copies per individual. Among the seven hatcheries, hatchery H_4 showed the lowest degree of WSSV infection

Table 2
Sampling information, biomass and WSSV infection of four wild *P. trituberculatus* populations collected from the Yellow Sea off the coast of Korea in 2007.

Population[a]	Sampling date	No. of specimens	CL (mm)	CW (mm)	BW (g)	Copies/ng DNA[c]	Copies/mg tissue[c]	Prevalence of positives (%)
P_J	Apr. 19	29	87.9 ± 6.5	184.8 ± 13.2[b]	364.9 ± 62.9	4.3 ± 3.8	1666.5 ± 1146.1	96.6
P_T	Jun. 30	20	86.9 ± 6.1	188.6 ± 11.9[b]	ND	3.2 ± 1.7	1091.7 ± 563.3	95.0
P_A	Aug. 22	120	69.1 ± 8.1	119.7 ± 14.4	186.6 ± 60.1	7.5 ± 7.1	2814.9 ± 2446.1	92.5
P_D	Dec. 24	53	81.1 ± 4.3	130.9 ± 6.7	244.9 ± 40.6	1.1 ± 1.9	1168.3 ± 2160.7	34.0
	Total	222				5.2 ± 6.1	2116.5 ± 2261.9	79.3

[a] P_J, Jindo population; P_T, Taean population; P_A, Anmyeondo population; P_D, Daecheon population.
[b] TCW (total carapace width).
[c] There was a significant difference in WSSV infection between any two populations ($P = 0.000$) except that between P_J and P_T ($P = 0.247$).