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Increasing Production in Korean Shrimp Farms with White-Spot Syndrome Virus PCR-Negative Brood Stock

SEOK, SEUNG HYEOK, MIN WON BAEK, HUI YOUNG LEE, DONG JAE KIM, MYUNG SUN CHUN[1], JONG SHEEK KIM[2], SE OK CHANG[3], AND JAE HAK PARK*

Department of Laboratory Animal Medicine, College of Veterinary Medicine and KRF Zoonotic Disease Priority Research Institute, Seoul National University, Seoul 151-742, Korea
[1]Dong-a Science, Science Culture Research Center, Seoul 120-715, Korea
[2]National Fisheries Research and Development Institute, Incheon 400-420, Korea
[3]Shin Chon Feed Co., Ltd., Incheon 404-260, Korea

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Abstract White-spot syndrome virus (WSSV) is a devastating, infectious virus affecting shrimp. Although sensitive techniques involving PCR have been developed to assist farmers in screening shrimp (brood stock) for WSSV prior to stocking ponds, such practices have not yet been applied in Korea. Despite the rationality of implementing screening, there has been some doubt as to whether the stocking of WSSV-PCR-negative fry epidemiologically decreases white-spot disease outbreaks. Here, we report a retrospective analysis of data from shrimp farms in the western coast of Korea where WSSV-PCR-negative brood stocks were used to stock rearing ponds. A total of 366 shrimp from Heuksan Island were sampled for WSSV with PCR. Of the tested shrimp, 7.2% (28 brood stocks) were identified as WSSV positive; only WSSV-PCR-negative shrimp were used for brood stocks. Total unit production (final shrimp production/ the area of the ponds) was higher, at 1.96, in ponds where WSSV-PCR-negative shrimp were used, as compared with 1.02 in other ponds in Korea in 2004. This retrospective analysis of WSSV in Korea may be useful to the shrimp aquaculture industry, suggesting a testable hypothesis that may contribute to the eventual control of WSSV outbreaks.

Keywords: White-spot syndrome virus (WSSV), PCR, VP28 envelope gene, shrimp

White-spot syndrome virus (WSSV) was discovered in 1992, and has since been recognized as a major cause of the devastation of shrimp production [3, 12]. In addition, the virus has spread quickly to most shrimp growing countries [5, 12, 19, 22]. Since 1993, WSSV has caused massive mortalities of the penaeid shrimp cultured in Korea [11, 20]; in particular, a major outbreak of WSSV in China in 1993 reduced shrimp production by 70% in a single year [1, 28].

The WSS virion is a bacilliform-shaped enveloped particle, approximately 275 nm in length and 120 nm in width [8], and contains a 300-kb circular double-stranded DNA molecule that comprises approximately 185 open reading frames (ORFs) [23], and the genome of WSSV was recently sequenced [23]. There have been few epidemiological studies of farmed shrimp performed [4, 16]; to date, there have been no studies of WSSV epidemics in Korea. WSSV has a broad range of hosts, infecting several crustacean species including shrimp, crab, and crayfish [25]. In shrimp, following oral infection with WSSV, infected cells are observed first in the stomach, gill, and cuticular epidermis; subsequently, the infection spreads systemically in the shrimp to other tissues of mesodermal and ectodermal origin [2].

Several nucleic acid and immunologically based detection protocols have been developed for this virus [7, 9]. One such detection method is PCR, which is both a powerful and accurate technique; PCR for WSSV is able to detect 1 pg of WSSV DNA after 30 cycles of amplification using a template comprised of total nucleic acid extracted from diseased shrimp with no signs of viral infection [9]. Sensitive techniques using PCR have been developed [15, 26] to assist farmers in screening *P. monodon* post larvae (PL) for WSSV before stocking of ponds, although this practice has not been carried out in Korea. Despite the rationality of this approach, there has been some doubt as to whether stocking of WSSV-PCR-negative shrimp epidemiologically decreases white-spot disease outbreaks.

*Corresponding author
Phone: 82-2-880-1256; Fax: 82-2-887-1257;
E-mail: pjhak@snu.ac.kr

Here, we report a retrospective analysis of data recorded from one commercial shrimp farm from the western coast of Korea, where WSSV-PCR-negative brood stocks were used to stock rearing ponds. The aim of the present study was to identify the relative advantage of crop success resulting from the use of WSSV-PCR-negative brood stocks.

A shrimp hatchery company located on the western coast of Korea was stocked with *Fenneropenaeus chinensis* (*F. chinensis*) brood stock from 10 farms from March to November of 2004. The farms comprised 10 earth-ponds of variable size, ranging from 1.3 to 6.6 hectares, which were used for the culture of *F. chinensis*. Similar to most shrimp ponds in Korea, the shrimp hatchery adopted the method of "semi-closed" cultivation, whereby water exchange was minimized in order to reduce the chance of introducing waterborne carriers of WSSV [10, 14]. When necessary, water exchange was carried out from a chloride disinfected (20 ppm) reservoir, which was considered relatively free of these viruses and their carriers. Despite these precautions, the farms did not employ crab exclusion fences around the ponds during the study interval, although several types of crabs are WSSV carriers [10, 14]; the practice of using such fencing is still uncommon in Korea. For stocking, brood stocks were purchased from commercial sources at Heuksan Island in Jeonnam Province, located on the western coast of Korea.

The presence of WSSV in individual brood stocks was screened by using a single-step PCR, as previously described by Seok *et al.* [21]. The 1st or 2nd pereiopod was collected and homogenized with a ceramic mortar in a twenty-fold volume of sterile PBS. The homogenate was centrifuged at 3,000 rpm for 10 min, and the supernatent was collected and stored at −70°C in 1-ml aliquots. PCR was performed to amplify the complete open reading frames (ORF) of VP28, a 28-kDa structural viral protein of WSSV. Primers for PCR were designed from nucleotide sequences in the GenBank/EMBL databases of WSSV (AF272979 for VP28) [21]. The PCR primers were as follows. VP28 upstream: 5'-CTC GTC ATG GAT CTT TCT TT-3'; VP28 downstream: 5'-CTC GGT CTC AGT GCC AGA GT-3'. Total DNA isolated from the brood stocks of *F. chinensis* in Korea was used as the template. Briefly, the homogenate was mixed with SNET lysis buffer (1:3 ratio) (20 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0; 400 mM NaCl; 1% (w/v) SDS; 1 mg/ml proteinase K) and incubated for 8 h at 55°C. DNA was extracted with phenol-chloroform, precipitated in isopropyl alcohol with sodium acetate, washed twice in 70% alcohol, and resuspended in TE buffer (pH 8.0). PCR reactions (100 μl) contained 2 μg of DNA, 100 pmol of each primer, 1×PCR buffer, 2 mM $MgCl_2$, 200 μM dNTP, and 2.5 units of Taq polymerase (PCR Core Kit, Boehringer Mannheim, Germany). Gene amplification reaction conditions were as follows: 1 cycle of 94°C for 7 min; 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 10 min. Amplified products were detected by electrophoresis of 20-μl aliquots on 1.2% agarose gels.

Brood stocking and raising were performed in 2004 between June and November. Whether WSSV-PCR-positive or -negative, all brood stocks were normal in gross appearance and behavior, and presented no evidence of WSSV infection by normal histological analysis. Total shrimp production data from Korean shrimp farms in 2004 were provided by the National Fisheries Research and Development Institute in Korea. Shrimp production data of the farms using WSSV-PCR-negative shrimp for the brood stocks in 2004 were recorded and analyzed by our research team through a process of interviewing farmers. The ponds supplied by the Korean shrimp stock company in 2004 were not randomly selected, owing to the limit of utility and the size of the ponds; nevertheless, the importance of obtaining random samples was emphasized. Among the tested ponds, there were no differences in environmental factors such as the use of crab exclusion fences, water exchange systems, or the method of chloride disinfection.

The results of this study clearly revealed the advantages associated with diagnostic WSSV-PCR screening of brood stocks of cultivation ponds. Table 1 shows the total shrimp production in Korean shrimp farms in 2004. Several factors were associated with the mortality of 33% [201,432/611,392; number of dead *F. chinensis* (thousand)/number

Table 1. Total shrimp production of Korean shrimp farms in 2004.

Location	No. of ponds	Total area of ponds (hectares)	No. of *F. chinensis* juveniles (thousand)	Mortality		Final production (tons)	Unit production (tons/hectare)
				No. of ponds	Total no. of dead *F. chinensis* (thousand)		
Incheon	29	130.10	22,750	13	8,350	44.60	0.34
Kyeongki	21	102.20	28,100	15	20,015	35.00	0.34
Chungnam	118	734.10	191,520	40	78,630	559.80	0.76
Jeonbuk	67	339.90	64,870	52	55,261	53.60	0.16
Jeonnam	233	1,025.80	304,150	34	39,176	1,675.00	1.63
Total	468	2,332.10	611,390	154	201,432	2,368.00	1.02[a]

[a]Final production (tons)/Total area of ponds (hectares).

of *F. chinensis* juveniles (thousand)]. A major factor responsible for approximately 67% of the mortality in the Korean shrimp industry in 2004 was WSSV infection, and other factors included bacteria, parasites, and other viral infections [18]. Thus, WSSV infection confers the greatest threat to the growth of the shrimp aquaculture industry in Korea. Even though other factors including bacteria, parasites, and other viral infections were not checked in detail, there were no differences between the tested ponds that used WSSV-PCR-negative shrimp for the brood stocks and other Korean shrimp farms in environmental factors such as the use of crab exclusion fences, water exchange systems, or the method of chloride disinfection. Therefore, the tested ponds were exposed to the other factors that affected shrimp production at the similar condition except for WSSV infection.

Unit production (final production/the area of the ponds) of shrimp was higher at Jeonnam Province than other regions (Table 1). This difference of unit production can be attributed to location, as the shrimp ponds in Korea were recently moved from other provinces on the western coast of Korea to Jeonnam Province (southwestern coast of Korea) in an attempt to escape WSSV. Therefore, the ponds in Jeonnam Province are the newest ponds and are associated with high shrimp production. Table 2 shows the regional shrimp production of the Korean shrimp stock company, which uses WSSV-PCR-negative shrimp for the brood stocks, determined by one-step PCR with VP28 primers [21]. A total of 366 shrimp from Heuksan Island were sampled for WSSV by diagnostic PCR. Of those, 7.2% (28 brood stocks) were positive for WSSV by PCR assay, and only WSSV-PCR-negative shrimp were used for brood stocks. An infection rate of 7.2% represented a significantly lower WSSV infection percentage compared with other Asian countries, India and the Philippines [17, 24]. Vasseharan and colleagues [24] have reported that in India, among 84 *P. monodon* brooders collected, 29 (35%) were positive for WSSV by PCR assay. Similarly, Magbanua *et al.* [17] revealed that in the Philippines, 34% of brooders were positive for WSSV by PCR assay. In Chungnam and Jeonnam provinces, the unit production of the Korean shrimp stock company, which utilized WSSV-PCR-negative brood stocks, was higher than that of the total of the other Korean shrimp farms, according to data in 2004 (2.20 *vs.* 0.76 at Chungnam and 1.96 *vs.* 1.63 at Jeonnam Province). However, in Incheon Province, a WSSV infection occurred on May 2004 in the ponds using WSSV-PCR-negative shrimp. Owing to the limited sample number (only one pond at Incheon), no statistical analysis was performed. In Tables 1 and 2, the total unit production (1.96 *vs.* 1.02) was higher in the ponds using WSSV-PCR-negative shrimp than the other ponds in Korea in 2004. WSSV can be vertically transmitted from WSSV-positive spawners to their offspring [6, 13, 15], and the screening selection of WSSV-negative brooders markedly reduced the chances of a subsequent outbreak of WSSV. The water quality of the intensive shrimp aquaculture in Korea is substantial. Aside from the surface and subsurface sanitization of freshwater, the loadings of solids were considerable when the cumulative impacts from water exchange during the growout cycle and pond drainage during harvesting were taken into account.

Table 2. Production levels of a Korean shrimp stock company in 2004.

Location	Pond name	Total area of ponds (hectares)		No. of *F. chinensis* juveniles (thousand)	No. of ponds	Mortality: Total no. of dead *F. chinensis* (thousand)	Final production (tons)		Unit production (tons/hectare)
Incheon	Yeongjong		1.40	550	1	550*	0		0
Chungnam	Sipalbong	3.30	11.90	1,100	1	N	11.60	26.20	2.20
	Eoeun	4.00		1,500	2	450*	11.50		
	Gunnam	4.60		1,000	4	550*	3.10		
Jeonnam	Shinan	2.60	17.60	880	1	880*	0	34.50	1.96
	Gundochomyeon	6.60		2,200	1	N	10.00		
	GunsoeuidoA	2.60		1,100	1	N	3.00		
	GunsoeuidoB	1.30		400	1	N	4.50		
	GunimjadoA	1.50		500	1	N	6.00		
	GunimjadoB	3.00		1,100	1	N	11.00		
Total			30.90	10,330	14	N		60.70	1.96[a]

[a]Final production (tons)/The area of total ponds (hectares).

N: not done and no symptoms of WSSV infection were observed.

*Total no. of dead *F. chinensis* (thousand): These dead shrimps were determined as WSSV infection by the WSSV diagnositic PCR with VP28 primer. These PCR tests were performed through the random sampling (about 0.01%) on dead shrimps from the shrimp ponds that showed that almost all of the shrimps in the pond died after the first dead shrimps were observed within 3 to 4 d.

In the present study, the main source of the decrease in shrimp production was WSSV infection, and the route of virus infection may have been through WSSV-infected brooders. However, the use of WSSV-PCR-negative brood in the shrimp aquaculture industry increased the unit of shrimp production in the southwestern coast of Korea in 2004. A recent study by Hossain *et al.* [6] revealed that the incidence of WSSV observed in brood stock in India is 50%, and that the actual incidence may be even higher. Therefore, uninfected shrimp populations are an important control for the study of WSSV infection. However, given the highly infectious nature of WSSV, such populations have historically been difficult to establish [8] and thus the use of WSSV PCR for selecting the WSSV-negative brood stocks was a very useful method in our shrimp culture system.

To date, there is a lack of information on the true status of shrimp viral diseases, such as WSSV, in Korea. Consequently, this may pose a great threat to the growth of the shrimp aquaculture industry. It is therefore imperative that screening and monitoring programs be continued in order to determine the prevalence of some of the highly pathogenic shrimp viruses emerging in the farms and hatcheries in Korea.

Epidemiological studies in aquaculture are scarce, and the present study describes both the problems encountered during the setup and design of a WSSV observational study and the solutions utilized to address these problems. This retrospective analysis of WSSV outbreaks in Korea may prove useful to the shrimp aquaculture industry by suggesting testable hypotheses that may contribute to the eventual control of WSSV outbreaks.

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