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White Spot Syndrome Virus in Penaeid Shrimp Cultured in Korea

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Abstract Because of the great concern over the possibility of contamination from the rod-shaped nuclear virus (PRDV) from Japan and white spot virus (WSSV) from Taiwan, most eggs used in Korean shrimp farms are currently obtained from local broodstock. In addition, the screening of imported broodstock for any viral presence at the National Fisheries Research and Development Institute is also mandatory. Nonetheless, massive mortality from white spot syndrome continues in Korea. In the present study, we present an improved PCR method to use tissue-extracted DNA instead of viral DNA extracted from a purified virus based on a sucrose density gradient, and produced results within 8 h. In 1998, this modified PCR method was able to detect that diseased *Penaeus japonicus* were infected only with PRDV, while *Fenneropenaeus chinensis* were infected with both PRDV and WSSV. In 1999, PRDV and WSSV were detected in *F. chinensis* with signs of infection, but not with WSSV alone.

Key words: *Fenneropenaeus chinensis*, PCR, penaeid shrimp, *Penaeus japonicus*, white spot disease (WSD), WSSV

The shrimp industry in Asia is experiencing a widespread crisis due to the outbreak of a serious viral infection that affects all known species of cultivated penaeid shrimp [1]. It is well documented that the principal clinical signs of this disease are the presence of white spots on the exoskeleton and epidermis, ranging from barely visible to 3 mm in diameter, and a reddish discoloration of the body due to an expansion of the cuticular chromatophores [13]. Non-occluded bacilliform viral particles have been observed under a transmission electron microscope (TEM) [5, 8, 15, 16, 17]. In infection trials, the virus has been demonstrated to be highly pathogenic to kuruma shrimp [19]. This bacilliform virus was originally named as a rod-shaped nuclear virus of *Penaeus japonicus* (RV-PJ) [8, 19] and later changed to PRDV [9], and white spot baculovirus (WSSV) [14, 22]. WSSV would appear to be closely related to the hypodermal and hematopoeitic necrosis baculovirus (HHNBV) [7], reported to be the pathogen of the explosive epidemic disease of prawn (EEDS) in China in 1993-1994 [3], and systemic ectodermal and mesodermal baculovirus (SEMBV) of the black tiger prawn *P. monodon* in Thailand [22, 23]. In Korea, bacilliform viral particles have been observed in diseased *Fenneropenaeus chinensis* by electron microscopy [12] and identified as PRDV by a PCR reaction [18]. Kim *et al.* [11] reported a partial genomic sequence of baculovirus associated with the white spot syndrome in *F. chinensis*, which was distinct from any other previously reported sequences. The Korean peninsula is surrounded by three seas (east, west, and south) and shrimp farms are located on the west and south coasts. The shrimp farms on the west coast cannot culture shrimp during the winter because of the low temperature. Therefore, shrimp eggs for hatching in March have been obtained from imported broodstock from countries such as Japan and Taiwan as well as from locally captured broodstock. This has raised great concern about viral contamination from these countries. Since 1999, it has been mandatory for imported broodstock to be screened for the presence of viruses at the National Fisheries Research and Development Institute. However, mass mortality from viral infection continued during 1999. Therefore, viral screening at the farm by rapid and reliable diagnostic tools needs to be undertaken to prevent any spread.

Accordingly, we developed a fast PCR method for PRDV and WSSV, and used this method to detect viruses in penaeid shrimp collected in 1998 and 1999.

MATERIALS AND METHODS

Source and Preparation of Specimens

The specimens of *F. chinensis* and *P. japonicus* were collected from three shrimp farms (Yongjongdo, Palkum,

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and Tae-an) located on the west coast of Korea. Each farm consisted of 20–40 hatchery, nursery ponds with 30–50 tons of sea water, and 20 rearing ponds of various sizes over approximately 8–16 acres. In 1998, *P. japonicus* of about 2 g and *F. chinensis* of about 2 g to 20 g bodyweight with signs of white spot syndrome were collected for study. In 1999, *F. chinensis* of about 2 g body weight were collected before/after the disease outbreak at intervals of 4–5 days. For histological examination, the cephalothorax was removed from each shrimp and fixed in Davidson's fixative for 1 week, then sagittally cut into 4 pieces from the base of the rostrum to the end of the cephalothorax. Each piece was dehydrated serially with alcohol and embedded in paraffin wax. The pieces were then cut into 3 μm sections and stained with hematoxylin and eosin (H&E).

Transmission Electron Microscope

The lymphoid organ was homogenized in phosphate buffer (pH 7.2) using a glass-teflon homogenizer and centrifuged at 1,000 ×g. The supernatant was negatively stained with 1% phosphotungstic acid and observed under a transmission electron microscope (JEM-100CX II, JEOL, Japan).

Preparation of Viral DNA from the Crude Tissue Homogenate

The shrimps were rinsed with a cold TE buffer (50 mM Tris, 1 mM EDTA, pH 7.6). In the case of adult shrimps (20 g), the tail muscle, total intestine, and pleopods were separately prepared. In the case of small shrimps, only the tail muscle and total intestine were prepared from five shrimps (2 g). Each part was soaked in a cold extraction buffer and homogenized in a bead beater (Biospec products, U.S.A.) with 20 rounds of a 20 s pulse and 20 s rest. After 30 min, NaCl (final conc., 1 M) was added to the homogenate and the mixture was kept in ice water for 1 h. The homogenate was then centrifuged at 1,000 ×g for 10 min. To extract DNA, the supernatant was incubated in a TE buffer containing 0.5% (W/V) sodium dodecyl sulfate and 1 mg/ml proteinase K at 65°C for 2 h and extracted with phenol-chloroform, followed by ethanol precipitation.

Amplification of PRDV and WSSV with PCR

The PCR reactions for PRDV and WSSV were performed as described previously (Park *et al.*, 1998) and the products were confirmed by agarose gel electrophoresis.

DNA Sequencing

PCR products were purified (Qiaquick gel extraction kit, Qiagen, Valencia, CA, U.S.A.). The sequencing PCR reaction was carried out with 1 μl (3–10 ng) of the purified DNA, 3.2 pmol of primer, and 3 μl of Big Dye Terminator according to the manufacturer's instruction (PE Applied Biosystems, Foster City, CA, U.S.A.). The extension product was precipitated with 95% cold ethanol after the addition of 3 M sodium acetate (final conc., 0.3 M), washed with 70% ethanol, dried in a speed-vac, and resuspended in 25 μl of Template Suppression Reagent. After denaturation at 95°C for 2 min, the sample was loaded onto a POP6 capillary column in a ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, U.S.A.).

Nucleotide Sequence Accession Number

The GenBank accession numbers of PRDV and WSSV are BankIt391338 AF361753 and BankIt391342 AF361754, respectively.

RESULTS

PCR using Viral DNA Extracted from the Purified Virus and the Crude Tissue Homogenate

When PCR was performed using WSSV-specific primers, PRDV viral DNA, PRDV primers, and WSSV viral DNA, both reactions failed to produce a band (Fig. 1). A PCR performed using PRDV or WSSV specific primers produced 643 bp or 1,447 bp fragments, respectively. DNA sequencing of the 1,447 bp product matched with the one reported by Lo *et al.* [14]. However, the DNA sequence of PRDV has not previously been reported, therefore its sequence was submitted to the Genbank. When PCR was performed with viral DNA purified from isolated viral particles, the whole procedure, including dissecting shrimp, virus purification, and PCR products analysis by agarose gel electrophoresis, took 2 days. However, when the DNA was purified from crude tissue homogenates, it took only 8 h. Moreover, shrimps could be kept alive when only the pleopods were used.

Detection of Virus

When shrimps with signs of infection, which were collected in 1998, were observed under a transmission electron microscope, viral particles were observed in the negatively stained homogenate of the lymphoid organ from every shrimp. All *P. japonicus* were found to be infected with

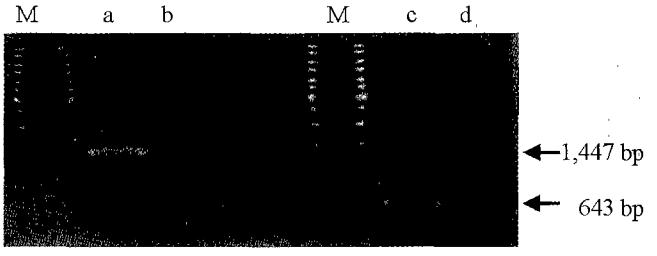

Fig. 1. PCR specific to PRDV and WSSV.
a: WSSV-specific primers and WSSV viral DNA; b: WSSV-specific primers and PRDV viral DNA; c: PRDV-specific primers and PRDV viral DNA; d: PRDV-specific primers and WSSV viral DNA; M: a 500 bp DNA ladder marker.

Table 1. Virus detection using PCR in shrimp collected from various shrimp farms on the west coast of Korea in 1998.

Sample				Signs[1]	PCR[2]	
No.[3]	Shrimp farm	Species	Part		PRDV	WSSV
1	Palkum	*P. japonicus*	Tail muscle	+	+	–
			Total intestine		+	–
2	Palkum	*P. japonicus*	Tail muscle	+	+	–
			Total intestine		+	–
3	Palkum	*P. japonicus*	Tail muscle	+	+	–
			Total intestine		–	–
4	Yongjungdo	*F. chinensis*	Tail muscle	+	+	+
			Total intestine		+	–
5	Yongjungdo	*F. chinensis*	Tail muscle	+	+	+
			Total intestine		–	–
6	Tae-an	*F. chinensis*	Tail muscle	+	–	+
			Total intestine		–	–
7	Tae-an	*F. chinensis*	Tail muscle	++	+	+
			Total intestine		–	–
			Tail muscle		–	+
8	Tae-an	*F. chinensis*	Total intestine	+	+	+
			Pleopods		+	+
			Tail muscle		+	+
9	Tae-an	*F. chinensis*	Total intestine	+	–	–
			Pleopods		+	+
10	Tae-an	*F. chinensis*	Tail muscle	–	–	–
			Total intestine		–	–

[1]Discoloration and white spot; [2]Primers specific to each virus; [3]Shrimp collected from different ponds.

PRDV, but not with WSSV. However, the *F. chinensis* were all infected with both viruses, with the exception of one (No. 6) that was only infected with WSSV. In the case of small shrimps, PRDV was detected in the tail muscles and intestines while WSSV was only detected in the tail muscles (Table 1). In the adult *F. chinensis*, both PRDV and WSSV were detected in the tail muscle, intestine, and pleopods.

When the tissue of *F. chinensis*, collected in 1999, was observed under a light microscope, no inclusion bodies were detected in the epithelia of the stomach or epithelia of the epithelium. Since it was difficult to obtain enough viral DNA from the pleopods because of their small size, viral DNA was prepared from the tissue homogenate of the tail muscle. Among sixteen groups of shrimps with viral signs, eight groups were infected with both PRDV and WSSV, seven were only infected with PRDV, and no shrimp was infected with only WSSV. Using PCR, no virus was detected in those shrimps with no viral signs, even though they were collected in the same pond only 4–5 days before the signs appeared (Table 2).

DISCUSSION

The early detection and prevention of the spread of viral diseases is very important in the shrimp industry, where there is no treatment after being infected. Viral diseases in shrimps can spread rapidly, killing every shrimp in a shrimp farm in less than 3–4 days. Various detection techniques have already been developed and used in fish farms [2, 4, 10, 20, 21]. Among them, PCR is the most sensitive and rapid detection method. However, the entire procedure, including dissecting the shrimp, virus purification, and analysis of the PCR products by agarose gel electrophoresis, takes two days. For faster detection, DNA prepared from a crude tissue homogenate was used and this method was found to be much faster than using the virus purified on a sucrose density gradient. The tissue homogenate can be prepared in less than 10 min. Moreover, virus detection without killing the shrimp is possible, particularly in the case of broodstock. As shown in Table 1, pleopods provide enough DNA for virus detection with a PCR. This modified method is superior to a PCR using DNA isolated from a purified virus in every aspect - time, ease of preparation, smaller sample volume, and no need to kill the shrimp. In particular, the tissue is homogenized in a mini-bead beater chamber and this produces a large number of samples that can be screened at the same time. However, this method was unable to detect any virus in shrimps which were collected 4-5 days before the disease outbreak and which did not display any signs of infection.

Table 2. Virus detection in *F. chinensis* collected at Daemyung shrimp farm in 1999.

Sample[1]		Signs[2]	LM[3]	PCR[4]	
No.	Part			PRDV	WSSV
1	Tail muscle	+	–	+	+
1-1[5]	Tail muscle	+	–	+	–
2	Tail muscle	–	–	–	–
2-1	Tail muscle	+	–	–	–
3	Tail muscle	–	–	–	–
3-1	Tail muscle	+	–	+	–
4	Tail muscle	–	–	–	–
4-1	Tail muscle	+	–	+	+
5	Tail muscle	–	–	–	–
5-1	Tail muscle	+	–	+	+
6	Tail muscle	–	–	–	–
6-1	Tail muscle	+	–	+	+
7	Tail muscle	–	–	–	–
7-1	Tail muscle	+	–	+	+
8	Tail muscle	–	–	–	–
8-1	Tail muscle	+	–	+	–
8-2	Tail muscle	+	–	+	+
9	Tail muscle	–	–	–	–
9-1	Tail muscle	+	–	+	–
9-2	Tail muscle	+	–	+	+
10	Tail muscle	–	–	–	–
10-1	Tail muscle	+	–	+	–
11	Tail muscle	–	–	–	–
11-1	Tail muscle	+	–	+	+
12	Tail muscle	–	–	–	–
12-1	Tail muscle	+	–	+	–
13	Tail muscle	–	–	–	–
13-1	Tail muscle	+	–	+	–

[1]Shrimp (average weight=1.6 g) collected in a different pond; [2]Discoloration and white spot; [3]Inclusion bodies observed with light microscopy; [4]Primers specific to each virus; [5]Shrimp captured in the same pond after 4–5 days.

Since imported broodstocks are mandatorily examined for the presence of viruses, shrimps must have been infected with viruses in their environment; for example, by pond water or insects, as suggested previously [4, 6]. Moreover, Lo *et al.* [15] suggest that infected egg cells are killed by the viruses before maturation, leaving little chance of vertical virus transmission. Also, the fact that some shrimps were infected with two viruses suggested that the viruses originated from a contaminated environment, and not from the broodstock. Since the water and insects on the shrimp farms were not screened, it is still unclear how the viruses infected the shrimp.

The biggest problem in virus detection is that a virus cannot be detected in shrimp before the disease outbreak. Perhaps this is because the virus multiplies so fast once shrimps are infected, and therefore, it is impossible to detect them before the disease outbreak. To overcome this problem, a more sensitive method to detect viruses in shrimps before signs of infection needs to be developed with an understanding of the infection route, so as to prevent catastrophic losses in the shrimp industry.

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