Control of Penaeid Acute Viremia (PAV) in *Penaeus japonicus*: Selection of Eggs Based on the PCR Detection of the Causative Virus (PRDV) from Receptaculum Seminis of Spawned Broodstock

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ABSTRACT — In order to prevent penaeid acute viremia (PAV) in seed production of kuruma prawn *Penaeus japonicus*, the presence of penaeid rod-shaped DNA virus (PRDV) in the ovary and receptaculum seminis was examined by polymerase chain reaction (PCR). The detection rate of PRDV was always higher in the receptaculum seminis than in the ovary. The prevalence of the virus in the receptaculum seminis became higher after spawning, especially in the spawners that required a longer period to induce spawning. PAV occurred in postlarvae cultured at Kamiura Station of Japan Sea-Farming Association in 1997 when segregation of spawners was made based on the results of PRDV detection from ovaries before spawning, whereas the disease did not occur in 1998 or 1999 when segregation of eggs was performed according to the result of virus detection from the receptaculum seminis after spawning. These results indicate that the selection of eggs should be done in the hatchery based on the results of PCR detection from receptaculum seminis of spawners after spawning.

Key words: PAV, WSS, PRDV, *Penaeus japonicus*, broodstock, receptaculum seminis, baculovirus

Serious mortalities due to a viral disease among populations of cultured kuruma prawn *Penaeus japonicus* have occurred in western Japan since 1993 (Nakano *et al.*, 1994), and the disease has become a significant obstacle in prawn culture. In Japan, this viral disease has been called penaeid acute viremia (PAV), and the causative virus was named as penaeid rod-shaped DNA virus (PRDV) (Inouye *et al.*, 1996). PAV, equivalent of white spot syndrome (WSS) in south-east Asian countries (Takahashi *et al.*, 1996; Lo *et al.*, 1996), has become the most serious problem not only in shrimp farming industry but also in shrimp hatcheries for sea-farming (Momoyama *et al.*, 1997; Satoh *et al.*, 1999). WSS has been reported in cultured kuruma prawn, black

tiger shrimp *P. monodon*, redbelt shrimp *P. penicillatus* and Chinese prawn *P. chinensis* (Chou *et al.*, 1995; Wongteerasupaya *et al.*, 1995; Peng *et al.*, 1995; Lightner, 1996).

The major infection route of PRDV in grow-out ponds is considered to be by horizontal transmission from diseased prawns to healthy ones (Nakano *et al.*, 1994). On the other hand, in seed production facilities PAV is shown to be transmitted vertically from brooders to their offsprings (Maeda *et al.*, 1998; Satoh *et al.*, 1999). This suggests the importance of eliminating PRDV-positive spawners in seed production. In 1997, segregation of PCR-positive spawners was made based on the results of PRDV detection from ovaries before spawning according to our previous results (Mushiake *et al.*, 1998). However, PAV occurred in postlarvae,

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which were obtained from brooders caught between July and August. Thus, it was found that the selection of spawners based on the detection of PRDV from ovaries before spawning was insufficient as a control measure of PAV. Therefore, a more appropriate organ for the PCR detection of the virus in spawners should be searched for more reliable selection of the spawners.

In the present study, we examined the receptaculum seminis, which preserves sperm obtained from males through copulation located on the ventral side at the center of the body in females, as well as the ovaries for the detection of PRDV before and after spawning. As a result, it was found that the vertical transmission of the virus can be effectively prevented by eliminating the contaminated eggs based on the PCR detection of PRDV from receptaculum seminis after spawning.

Materials and Methods

Broodstock and spawning

Adult kuruma prawn, which were caught in coastal waters off eastern Kyushu, were purchased from dealers and used as spawners between April and August in 1997, or between March and July in 1998 and 1999.

In 1997, the ovaries (0.1 g) of all females (1,413 in total) were sampled individually by using a disposable syringe (3.0 mL; needle 19 G) and the stages of ovarian maturation were examined by biopsy (Miyajima and Matsumoto, 1996) just after transport to the Kamiura Station, Oita Pref., of Japan Sea-Farming Association (JASFA). At the same time, the receptaculum seminis (0.1 g) was sampled from some spawners before spawning from June. After individual microscopic examination of ovary, the spawners which reached the final maturational stage (oocytes with cortical alveoli) were selected and stocked individually in 10 L tanks at 15–18°C for spawning suppression until the PCR examination of PRDV from ovaries was completed. Only spawners (969 in total) having PCR-negative ovaries were installed into a spawning tank (25 m³) at 23–27°C for induction of spawning.

In 1998 and 1999, a total of 1,212 and 715 kuruma prawns were purchased, respectively. After transport to the station, 10 to 15 individuals without biopsy were stocked separately in 0.5 m³ spawning tanks at 23–27°C for induction of spawning. At the same time, ovaries (0.1 g) and receptaculum seminis (0.1 g) were sampled from some spawners before spawning.

Each spawning experiment was continued for 3 days in every year. After spawning in the first day, spawners were divided into 3 groups according to spawning states by perspective observation of the ovary; complete spawning, partial spawning and no spawning groups. From the spawners of the first and second groups, both ovary and receptaculum seminis were sampled for the PRDV detection by PCR, whereas

prawns with non-spawning were once again submitted to induction of spawning in the next day. The ovary and receptaculum seminis were sampled on the next day from the prawns with complete or partial spawnings, and the 3rd group was passed on to the next day. The same procedure was repeated in the 3rd day. From the prawns which did not spawn even in the 3rd day, both tissues were sampled on the 4th day. Based on the PCR results on the receptaculum seminis after spawning, the eggs from PCR-negative spawners were used in the following rearing experiments.

Rearing and sampling of seedlings

In 1997, the eggs just after obtained from PCR-negative brooders were disinfected with povidone-iodine (final concentration was 5 mg/L as active ingredient) for 5 min, and stocked in rearing tanks (100 or 150 m³) kept at temperature between 23 and 27°C. In 1998 and 1999, the eggs from every batch of spawners were disinfected as previous year and stocked in 0.2 m³ tanks until the PRDV detection was completed. And only eggs from PCR-negative spawners were stocked in rearing tanks (100 or 150 m³).

During the rearing experiments in these 3 years, the larvae were fed *Tetraselmis tetrathele*, nauplii of *Artemia salina* and commercial formula feed, supplying sand-filtered and ozone-disinfected sea water maintained at 23°C or higher. Kuruma prawn seedlings were sampled at the nauplius, zoea, mysis and postlarval stages (P1: 1 mg of average body weight, P5: 1.5 mg, P10: 3 mg, P20: 12 mg, P30: 30 mg, P40: 100 mg and P50: 200 mg). Each developmental stage: P1, P5, P10, P20, P30, P40 and P50 indicates 1, 5, 10, 20, 30, 40 and 50 days post-metamorphosis, respectively, as usually termed in kuruma prawn seed production facilities in Japan.

DNA extraction and detection of PRDV by PCR

The sampled ovary and receptaculum seminis were homogenized and digested with ISOGEN (Japan Gene Company). The total DNA was extracted with chloroform and ethanol and two PRDV specific primer sets, P1/P2 and P3/P4, were used as reported by Kimura *et al.* (1996). After 30 cycle amplification for each primer set at 93°C (60 s), 57°C (90 s) and 72°C (60 s), amplified products were analyzed by agarose gel electrophoresis. PRDV-infected *P. japonicus* was similarly processed as a positive control.

Results

Detection of PRDV before and after spawning

The prevalence of PRDV from ovary and receptaculum seminis of spawners before and after spawning from 1997 to 1999 are shown in Table 1. The total prevalence of PRDV from the ovaries of broodstock before

Table 1. Prevalence of PRDV from ovary and receptaculum seminis of kuruma prawn spawners by nested PCR before and after spawning during 1997–1999

Date of purchase	Prevalence of PRDV (%)			
	Ovary		Receptaculum seminis	
	pre-spawning	post-spawning	pre-spawning	post-spawning
'97. Apr.	1.6 (4/248)* ¹	3.4 (3/ 87)	NE* ²	NE
May	0 (0/ 81)	0 (0/ 40)	NE	NE
Jun.	0 (0/108)	0 (0/ 37)	0 (0/ 38)	2.3 (2/ 86)
Jul.	0.7 (2/297)	4.0 (4/101)	8.6 (6/ 70)	39.2 (83/212)
Aug.	1.3 (3/240)	10.0 (10/100)	5.7 (2/ 35)	52.6 (41/ 78)
Total in 1997	0.9 (9/974)	4.7 (17/365)	5.6 (8/143)	33.5 (126/376)
'98. Mar.	0 (0/ 3)	0 (0/ 18)	0 (0/ 3)	0 (0/ 18)
Apr.	0 (0/ 38)	0 (0/587)	0 (0/ 38)	1.4 (8/587)
May	0 (0/ 13)	0.9 (1/111)	0 (0/ 13)	0.9 (1/111)
Jun.	0 (0/ 13)	1.6 (2/122)	0 (0/ 13)	13.9 (17/122)
Jul.	0 (0/108)	1.4 (2/148)	0 (0/ 21)	24.3 (36/148)
Total in 1998	0 (0/175)	0.5 (5/986)	0 (0/ 88)	6.3 (62/986)
'99. Mar.	0 (0/ 15)	0 (0/181)	0 (0/ 15)	0 (0/181)
Apr.	0 (0/ 10)	0 (0/262)	0 (0/ 10)	0 (0/262)
May	0 (0/ 5)	0 (0/ 39)	0 (0/ 5)	0 (0/ 39)
Jun.	0 (0/ 10)	6.8 (5/ 74)	0 (0/ 10)	41.9 (31/ 74)
Jul.	0 (0/ 7)	10.6 (7/ 66)	14.3 (1/ 7)	56.1 (37/ 66)
Total in 1999	0 (0/ 47)	1.9 (12/622)	2.1 (1/ 47)	10.9 (68/622)

*¹ (PCR positive/examined).*² Not examined.

spawning was 0.9% in 1997, and 0% in 1998 and 1999. The values for ovary after spawning were 4.7% (1997), 0.5% (1998) and 1.9% (1999). From the receptaculum seminis, the total prevalence of PRDV before spawning was 5.6% in 1997, 0% in 1998 and 2.1% in 1999, whereas after spawning the values were 33.5, 6.3 and 10.9%, respectively. Thus, PRDV was detected from the receptaculum seminis at higher rate after spawning than those before spawning and its prevalence increased rapidly from June on.

Prevalence of PRDV in different spawning states

Figure 1 shows the prevalence of PRDV from receptaculum seminis depending on the spawning state of broodstock after spawning. In 1997, the prevalence of PRDV in July and August were 44.1% and 61.9% in the group of complete spawning (Group 1), and these values were higher than those in the groups of partial spawning (Group 2) or non-spawning brooders (Group 3). In Group 1, these values in June and July in 1998 were 15.7% and 25.3%, whereas those in 1999 were 64.7% and 90.7%, respectively. Thus, brooders with complete spawning showed the highest prevalence of PRDV among 3 groups.

Prevalence of PRDV in 3 groups with different induction periods

The prevalence of PRDV in the receptaculum seminis after spawning was compared among the 3

groups with different induction periods (1-3 days) for spawning as shown in Fig. 2. In 1997, the prevalence of PRDV on the 3rd day post-spawning in July and August were 83.8% and 91.7%, respectively. The values in June and July were 100% and 92.3% in 1998, and 90.0% and 100% in 1999.

Discussion

As reported previously, PRDV was detected at higher rates from the ovaries than from the hemolymph or stomach in wild-captured female broodstock (Mushiaki *et al.*, 1998). On the basis of these results, we had attempted to prevent PAV in seed production in 1997 by selection of PRDV-negative spawners based on the PCR detection of the virus from ovaries just before spawning. However, PAV occurred in post-larval juveniles obtained from PCR-negative brooders caught between July and August, in the rearing experiments at Kamiura Station of JASFA. In the present study, it became evident that the detection rate of PRDV from the receptaculum seminis of prawns after spawning was much higher than that from the ovaries before spawning as shown in Table 1. Based on these results, only eggs obtained from PCR-negative in the receptaculum seminis were supplied for rearing experiments in 1998 and 1999. As a result, PRDV was not detected from juveniles in any developmental stages up to P50 and no PAV infection occurred during the rearing experiments

Fig. 1. Prevalence of PRDV in the receptaculum seminis of kuruma prawn spawners at different spawning states during 1997–1999.

*Not examined.

▨ complete spawning, □ partial spawning, ■ non-spawning

Fig. 2. Prevalence of PRDV in the receptaculum seminis of kuruma prawn spawners of 3 groups with different induction periods (1–3 days) for spawning during 1997–1999.

*Not examined.

▨ 1st day, □ 2nd day, ■ 3rd day

carried out in these 2 years. Similarly, in large scale seed production (200–2,500 m³) of kuruma prawn at Shibushi Station of JASFA (Kagoshima Pref.), no PAV occurred in juveniles up to P40–P65 (1998, 1999) after adopted the same method of control. These results strongly indicate that the selection of eggs based on the PRDV detection from the receptaculum seminis of brooders after spawning is a practical way of controlling PAV in seed production.

The present study also demonstrated that the detection rate of PRDV in the receptaculum seminis was higher when the females completely spawned, especially in the females which required longer incubation period for spawning. This suggests that spawning stress is the critical factor to allow viral multiplication in broodstock. However, we cannot conclude whether the PRDV detected in receptaculum seminis originated from the sperm of the male or from some organs of the female. In *P. monodon*, Lo *et al.* (1997) reported that WSBV (white spot syndrome baculovirus) was highly

detected in both male and female brooders, and that WSBV was present in the muscle and connective tissue of receptaculum seminis by *in situ* hybridization, but was absent in the sperm and epithelial cells of the organ. These results seem to indicate that the higher detection rate of WSBV from the receptaculum seminis in *P. monodon* was caused by the viral transmission from male to female during copulation. However, our previous report showed that the prevalence of PRDV in wild-captured adult female kuruma prawns was higher than that in male prawns (Mushiake *et al.*, 1998). Detailed information on the increase of PRDV in the receptaculum seminis of *P. japonicus* for the progress of spawning will be given by a study using *in situ* hybridization.

The increase in the detection rates of PRDV from receptaculum seminis or ovaries after spawning in kuruma prawns corresponds with the result of previous work on *P. monodon* (Lo *et al.*, 1997). The increase in detection rate of PRDV in the receptaculum seminis is

caused by the multiplication of the virus during the spawning, probably due to stresses from handling and spawning, or by the reduction in the amount of sperm, which will disturb PCR reaction with insufficient DNA extraction.

In *P. monodon*, it was suggested that infected egg cells are killed by WSBV before maturation (Lo *et al.*, 1997), and that rinse or disinfection of the nauplii is one of the most effective way as a preventive measure of the disease in seed production (Lo and Kou, 1998). However, in *P. japonicus*, it was found that disinfection of the nauplii with povidone-iodine gave a serious damage to their survival (data not shown). Therefore, in the present study, eggs were disinfected with povidone-iodine (5 mg/L as active ingredient for 5 min) in order to inactivate the virus that might attach to the surface of the eggs. The effectiveness of this method was not approved because PAV occurred in juveniles from the infected eggs in 1997.

The egg collection of kuruma prawn for seed production completely depends on wild-captured broodstocks in Japan. As shown in Table 1, the prevalence of PRDV increased rapidly from June. Therefore, it is also an effective measure for controlling PAV to use broodstocks caught before June for seed production. Trials of egg collection from artificially reared *P. japonicus* have been made, however, it has been pointed out that these eggs were of an inferior quality to those of wild-captured broodstock (Yano, 1987; Y. Miyajima, unpublished data). Therefore, at present, to use wild-captured kuruma prawn adults after segregation of PRDV-positive spawners is only the practical option for seed production of this species in Japan.

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