Preparation of an Inoculum of White Spot Syndrome Virus for Challenge Tests in *Penaeus japonicus*

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ABSTRACT—An inoculum of white spot syndrome virus (WSSV), called penaeid rod-shaped DNA virus (PRDV) in Japan, was prepared for challenge tests in kuruma shrimp *Penaeus japonicus*. The hemolymph was drawn with PBS from moribund shrimp, which were intramuscularly (IM) inoculated three days before with a virus suspension prepared from naturally affected shrimp. The virus concentration in the hemolymph was quantified as $1.5 \times 10^7$ genome copies/µL hemolymph by competitive PCR. The LD₅₀ of the inoculum prepared from the hemolymph stored at –80°C for 40 days was calculated by IM challenges as $10^{-4.2}$ µL hemolymph/g shrimp or $9.5 \times 10^2$ genome copies/g shrimp. Virulence tests were also conducted through IM injections with the virus preparation stocked for 5 and 16 months. As a result, there was no significant difference in cumulative mortalities ($p > 0.05$) among the 3 challenge tests made after preservation of the virus in the hemolymph at –80°C for 40 days, 5 or 16 months.

Key words: white spot syndrome virus, inoculum, *Penaeus japonicus*, virulence, challenge test, penaeid acute viremia, WSSV, PAV

White spot syndrome (WSS) has severely affected shrimp farming industry worldwide since it emerged first in Asia around 1993 (Lightner, 1996). Geographic isolates of the causative agent, commonly known as WSS virus (WSSV), from penaeid shrimp have proved homogeneous in both structural protein profiles and gene sequences although some differences were found between crayfish (*Orconectes punctimanus*) and penaeid shrimp isolates (Wang et al., 2000a, b). The taxonomy of the virus is presently unassigned but recent informations on structural proteins and genes show that the virus possibly belongs to a new family (van Hulten et al., 2001). Studies on the host-virus interaction have shown inducible resistance in kuruma shrimp which was supported by humoral neutralizing factor(s) against WSSV (=penaeid rod-shape DNA virus) (Venegas et al., 2000; Wu et al., 2002).

Various studies like these have been made on WSS or WSSV, however, challenge tests with quantified inocula of the virus have never been conducted due to the lack of susceptible cell line for the virus. In the present study, a virus inoculum was prepared by collecting the hemolymph from experimentally infected shrimp, quantified its gene copies by a competitive PCR, and tested on its virulence in *P. japonicus*. As a result, it was confirmed that the prepared inoculum provided stable virulence of the virus even after a storage at –80°C for 16 months. This made possible the monitoring experiment on the acquired resistance of *P. japonicus* against WSSV as reported in our previous paper (Wu et al., 2002)

Materials and Methods

Shrimp

WSSV-free wild adults and cultured juveniles of
Changes in viral genome titres (VGT) of WSSV in hemolymph

To know an appropriate time for collecting hemolymph from shrimp, changes in the number of WSSV genomes in the hemolymph of shrimp inoculated with different doses of WSSV were monitored at different time points by PCR analysis, as follows: Twenty-five adult shrimp (mean body weight, MBW: 100 g) were randomly divided into 5 groups (n=5) and reared in five 200 L tanks with sand beds. Shrimp in the groups 1–4 were intramuscularly (IM) injected with virus suspensions (Lot O virus, prepared from naturally infected shrimp) at doses of 10⁻³, 10⁻⁵, 10⁻⁷ and 10⁻⁹ μL/g shrimp, respectively. The remaining shrimp (group 5) were injected with PBS as control. Prior to injection, 0.1 mL of hemolymph was withdrawn from ventral artery in the 5th segment of individual shrimp using a 1 mL-disposable syringe filled with 0.5 mL cooled phosphate-buffered saline (PBS). After injection, the hemolymph collection was done everyday in the first week and every other day in the second week from each shrimp. Each collected hemolymph sample was stored at −80°C in 0.2 mL aliquots in triplicates for subsequent determination of viral genome titer (VGT).

To determine VGT, an aliquot of the hemolymph was thawed and centrifuged at 30,000 × g for 1 h at 4°C. The resultant pellet was resuspended in 300 μL PBS, then treated with 35 μL proteinase K (10 mg/mL) and 35 μL SDS (10%) at 37°C for 30 min. The DNA was extracted twice with TE-saturated phenol (TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and once with chloroform/isoamylalcohol (24:1). The supernatant was then added with 5 M acetic ammonium (pH 5.2), and DNA was recovered by ethanol precipitation at −80°C for 1 h, and dissolved in 100 μL TE. Tenfold serial dilutions of the DNA were subjected to PCR amplification (30 cycles) with primers P1/P2 (Kimura et al., 1996) and products were analysed with 1% agarose gel electrophoresis. The exponential value of the end-point dilution of DNA solution showing a visible band in agarose gel after amplification was used as VGT. In the case of the original DNA solution failed to present a visible band, VGT was expressed as − (negative).

Viral titers in hemolymph assessed by competitive PCR

Some 10 adult shrimp (MBW: 150 g) were IM inoculated with Lot O virus at a dose of 10⁻³ μL/g shrimp, and hemolymph was collected with 4-fold cooled PBS from 3 moribund shrimp on the third day post-inoculation. The
Results and Discussion

Changes in VGT in hemolymph

The VGT values tended to increase in the hemolymph of shrimp after inoculation with a dose of $10^{-3}$ or $10^{-5}$ μL/g shrimp of Lot O virus preparation, the values reaching 2 to 4 when the shrimp became moribund 3-4 days after inoculation (Table 1). Cumulative mortalities in the challenged groups with these 2 higher doses ($10^{-3}$ and $10^{-5}$ dilutions) reached 100%. Therefore, in the next experiment to obtain a virus inoculum for challenge tests, the hemolymph was collected on the third day post-inoculation.

DNA subjected to PCR amplification in VGT tests was extracted from pelleted virus after centrifugation of the sampled hemolymph. When, in a preliminary test, DNA was directly extracted from the hemolymph without purification of the virus by centrifugation, successful amplification was not attained. This may be due to the presence of an inhibitor(s) of PCR in the hemocytes or hemolymph.

WSSV infects many target organs of ectodermal and mesodermal origin and thus causes a systemic infection in shrimp (Wongteerasupaya et al., 1995). Owing to an open circulating system, the hemolymph of infected shrimp consequently contains lots of virus particles as Momoyama et al. (1995) observed by dark-field microscopy, hence often used as a source for virus purification (Kimura et al., 1995). In addition, hemocytes have been shown to be one of the target cells for WSSV (Durand et al., 1996; Wang et al., 1999). The hemolymph can also be obtained from moribund shrimp without intense bacterial contamination, thus harvesting hemolymph from moribund shrimp is a convenient method for obtaining large quantities of the virus.

Viral titers measured by competitive PCR

The number of virus genomes in Lot A hemolymph was calculated by competitive PCR as $5.8 \times 10^6$ copies/μL extracted DNA solution or $1.5 \times 10^7$ copies/μL hemolymph (Fig. 1).

Virulence of inoculum virus

Cumulative mortalities in the shrimp groups 1-5 inoculated with 5 serial dilutions ($10^{-1.17}$ to $10^{-5.17}$ μL hemolymph/g shrimp) of Lot A hemolymph stocked for 40 days were 100, 92, 88, 68 and 0%, respectively. LD$_{50}$ was calculated to be $10^{-4.16}$ μL hemolymph/g shrimp (Spearman-Karber method), or $9.5 \times 10^2$ genome copies/g shrimp.

In the virulence tests of the preserved virus (Fig. 2), high cumulative mortalities (88, 76, 68%) were observed

Table 1. Changes in viral genome titers (VGT*) in the hemolymph of Penaeus japonicus injected intramuscularly with WSSV at 10−3, 10−5, 10−7 and 10−9 dilutions of a virus preparation (Lot O)

<table>
<thead>
<tr>
<th>Doses and the No. of shrimp individual</th>
<th>VGT</th>
<th>Days after injection</th>
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<tr>
<td></td>
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<td>0 1 2 3 4 5 6 7 8 9 10 11</td>
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<tr>
<td>10⁻⁹ dilution</td>
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<td>10⁻³ dilution</td>
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*VGT: Exponential highest test showing positive PCR
** - -: The original DNA solution failed to present a visible band
D: Dead; NT: not tested
in the 3 test groups inoculated with almost the same inoculum volume per gram shrimp of the same virus preparation at 40 days, 5 months and 16 months after preservation, no significant differences in mortalities (χ² test, p>0.05) being observed among the tests. Dead shrimp in these groups were all positive for first-step PCR for WSSV and no mortality was observed in the control groups. The result indicated that the virus originally collected from the hemolymph of moribund shrimp could maintain its virulence for at least 16 months at -80°C. ln our preliminary experiments virus inocula prepared from 0.45 μm-filtered homogenates of infected post-larval cadavers lost its virulence when preserved for 3 weeks at -80°C and virulence of the virus in stored whole shrimp cadavers at -80°C also decreased gradually with time (data not shown). However, Momoyama et al. (1998) showed that the virulence was kept for 14 months at -80°C in a filtered homogenate prepared from moribund shrimp with hepatopancreas and abdomen removed. Therefore, if shrimp big enough to obtain the hemolymph are not available, this method of Momoyama et al. can be used.

References


Wang, Y. G., M. D. Hassan, M. Shariff, S. M. Zamri and X. Chen (1999): Histopathology and cytopathology of white spot syndrome virus (WSSV) in cultured Penaeus monodon from Peninsular Malaysia with emphasis on pathogenesis

