Infectivity of White Spot Syndrome Virus (WSSV) to the Polychaete *Pereneis nuntia* and a Possibility of WSSV Transmission from the Polychaete to the Black Tiger Shrimp *Penaeus monodon*

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ABSTRACT

Polychaetes are one of the live feeds for broodstock of Penaeus monodon, especially for the domesticated broodstock. Their contents are rich in arachnidonic acid, eicosopentaenoic acid and dihydroxyhexaenoic acid and are believed to help increase the fecundity of the broodstock. It has been of some concern that polychaetes may contain white spot syndrome virus (WSSV), which could be transmitted to the broodstock and subsequently to the postlarvae. The purpose of this study was to determine if the polychaete, *Pereneis nuntia*, could be infected with WSSV and, if so, whether it could transmit the virus to the shrimp. The study was divided into three experiments. In the first experiment, 240 wild *P. nuntia* were stocked in four 70 L aquariums, with 60 polychaetes in each aquarium. At day 0, three polychaetes from each aquarium were checked for the presence of WSSV by using nested polymerase chain reaction (PCR). Eight out of 12 polychaetes were found to have light-positive reactions. The polychaetes were fed for one day with meat from WSSV-infected *P. monodon*, and randomly sampled for PCR detection of WSSV, as well as for histology to search for any evidence of cellular changes caused by viral infection. From day 1 to 60, results from the PCR revealed variable proportion of positive cases, from 1/12 to 11/12, and most of them were very lightly or light-positive. Moderate to severe infections occurred only during the first two weeks following WSSV inoculation. Under light microscopy, no cells with hypertrophic nuclei typical of WSSV infection were detected in any of the polychaetes, even with severe WSSV infection. In the second experiment, healthy *P. monodon* free of WSSV infection, were fed with PCR-positive polychaete and the survival rate and development of white spot syndrome (WSD) was compared with control shrimp,

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which received either pellets or PCR-negative polychaetes. The shrimp fed on PCR-positive polychaete had survival rate comparable to that of the control groups and did not develop WSD, as confirmed by PCR and histology. In the third experiment, 5 WSSV-free shrimp were stocked together with 80 polychaetes in an aquarium and WSSV was added into the water. The shrimp developed WSD and died. One week later, 10 new WSSV-free shrimp were stocked into the aquarium and no mortality was observed; they were also PCR-negative and had normal histology. The polychaetes were light- or very lightly-positive with proportion varying from 3/10 to 9/10 within the 42 day-period. Results from the three experiments suggest that WSSV that enters the polychaete, *P. nuntia*, may not be able to replicate and remains in such a low amount, or becomes attenuated to the point that it cannot infect P. monodon.

INTRODUCTION

Polychaetes are the most morphologically diverse class of the Phylum Annelida with over 5,000 species. They occupy every part of the marine ecosystem but are especially abundant in the littoral zone. They are segmented marine worms and are extensively used as bait in angling. Polychaetes contain high amounts of essential polyunsaturated fatty acids, especially arachnidonic acid, eicosopentaenoic acid and dihydroxyhexaenoic acid (Graeve *et al*., 1997; Luis and Passos, 1995; Millamena *et al*., 1986), which are believed to help increase the fecundity of shrimp broodstock. Therefore, live polychaete feed is recommended for shrimp broodstock diets (Harrison, 1991) especially in Penaeus monodon broodstock (Chunhabundit, 1991; Tandavanitj and Kaowtapee, 2000). There are many kinds of polychaete species which have been found in many parts of Thailand. *Perineresis aibuhitensis, P. quatrefagesi, P. singaporiensis, P. striolata* and *P. nuntia* are found along the coast of the Andaman Sea; and *P. vacaurica* in the Gulf of Thailand. The polychaete, *P. nuntia*, is probably the most abundant species found in Thailand and has been widely used in commercial shrimp hatcheries in the country.

White spot syndrome virus (WSSV) is the causative agent of white spot disease (WSD), the disease that causes outbreaks and mass mortality in several shrimp species and in many countries worldwide. The virus infects a broad host range, including wild and farmed shrimp (for review, see Flegel, 2001). Since polychaetes are one of the important live feeds for broodstocks, it is therefore necessary to determine if polychaetes are WSSV carriers. There has been some concern that polychaetes (*Perinereis* spp.) in shrimp ponds and in natural environment that were found positive by PCR specific for WSSV (Ruangsri and Supamattaya, 1999; Tandavanitj and Kaowtapee, 2000) could transmit the virus to the broodstocks and subsequently to the postlarvae (Withyachumnarnkul, 1999). The purpose of this study was therefore to determine if polychaetes could be infected with WSSV and whether they could transmit the virus to the shrimp.

MATERIALS AND METHODS

This study was divided into three experiments. All the aquariums used in this study were placed in a closed room with dim light and ambient temperature of 28-30°C. In the first experiment, 60 wild *P. nuntia* (8-10 cm long) were stocked in four 70 L aquariums. The aquaria were lined with 30 cm deep-sand; the sand was lifted approximately one inch above the aquarium floor by a plastic sheath and net so that there was a narrow water column between the sand and the aquarium floor (Fig. 1). The purpose of this set-up was to aerate and drain the water. The tanks were filled with clean seawater (35 ppt) to approximately 1 cm above the sand during feeding time, which was at 08.30 h and at 16.30 h, initiating the "high-tide" periods. After one hour of feeding, the water was drained to about one-third of the sand level, initiating the "low-tide" periods. Therefore, most of the time, the sand was not covered with seawater. Using this set-up and procedure, the polychaetes could be kept healthy throughout the whole 60 day experimental period.

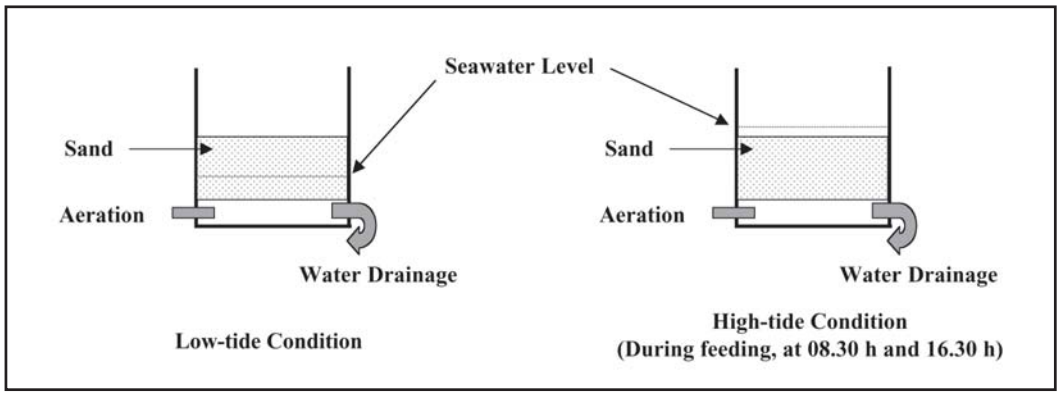

Figure 1. Diagram showing a polychaete aquarium. The 70 l-aquarium was lined with 30 cm deep-sand; the sand was lifted approximately one inch above the aquarium floor by a plastic sheath, for aeration and water drainage. At the simulated low-tide, the water was leveled down to about one-third of the sand bottom level; and at the simulated high-tide, the water was about 1 cm above the sand level.

The polychaetes were fed with meat from WSSV-infected *P. monodon*, which had been experimentally infected by WSSV injection, for three days. The shrimp were proven to have WSSV infection by demonstrating positive reactions with a nested-PCR IQ2000 WSSV Detection System (Farming Intelligence Technology Corporation, Taipei, Taiwan). Three polychaetes from each aquarium were then randomly sampled at day 0 (before feeding with WSSV-infected shrimp meat), 4, 7, 10, 13, 16, 19, 22, 30, 35, 41, 50 and 60. The appearance of bands specific for WSSV in an agarose gel plate was semi-quantitatively graded as negative, very lightly, light, moderate and severe WSSV infections according to the presence of reaction bands (Fig. 2). DNA sequencing of the 296 bp PCR product from random samples of three polychaetes with light infection was confirmed for the specificity of WSSV detection.

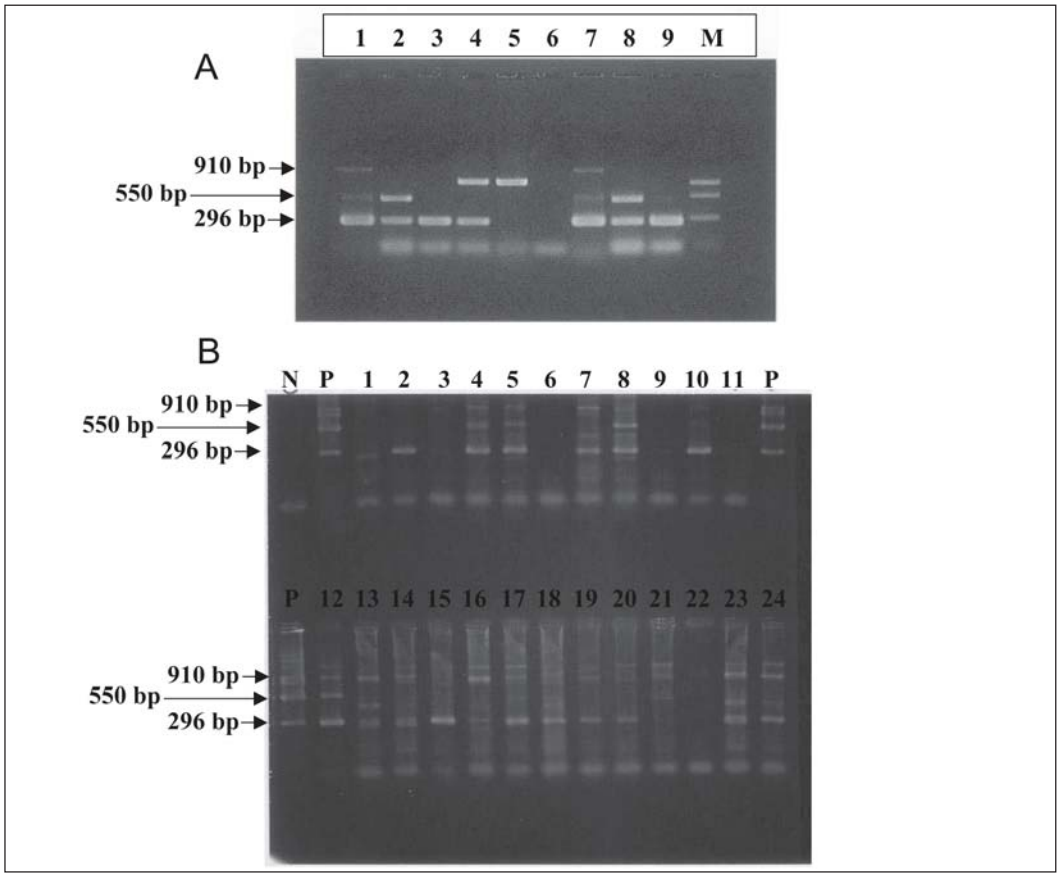

Figure 2. Panel A. Agarose gel electrophoresis showing bands of PCR products according to nested-PCR IQ2000 WSSV Detection System from Farming Intelligene Technology Corporation, Taipei, Taiwan. Lane 1, severe WSSV infection; lane 2, moderate WSSV infection; lane 3, light WSSV infection; lane 4, very light WSSV infection; lane 5, negative control; lane 6, water; lane 7, standard 1 - 2,000 copies/reaction; lane 8 , standard 2 - 200 copies/reaction; lane 9, standard 3 - 20 copies/reaction; lane M, markers (848 bp, 630 bp, 333 bp). Interpretation of the stage of WSSV infection is based on a combination of the three PCR product bands (296 bp, 550 bp and 910 bp). Panel B. Agarose gel electrophoresis of some polychaete samples and positive controls of WSSV genome. Lane N, negative control; lanes P, positive control; lanes 1-24, polychaete samples.

Polychaetes that were found to be severely infected by nested PCR were processed for histology, with hematoxylin and eosin staining. Light microscopic examination focused on signs of WSSV infection in cells of ectodermal and mesodermal origins, especially examining for the presence of hypertrophic nuclei of epithelial cells. The hypertrophic nucleus is a typical histological feature of WSSV-infected cells (Flegel *et al*., 1997).

In the second experiment, polychaetes from the first experiment at day 30 post-WSSV inoculation, that were PCR-positive and-negative, were fed to healthy, specific pathogen-free (SPF) including WSSV-free, domesticated *P. monodon* (Withyachumnarnkul *et al*., 2001), 5-8 g BW, for three days. The reason for using the polychaetes at day 30-post inoculation was to ensure that WSSV that resides in the polychaetes were those that were inside the polychaete cells, not those that were located extracellularly, for instances, in the

gut lumen or in the sand or water. WSSV survives only a few days extracellularly (Maeda *et al*., 1997) and 30 day-period was long enough to exclude any possibility that subsequent infection of *P. monodon* might come from inoculated WSSV that were located extracellularly. The shrimp were divided into three groups: the first group was fed with normal pellets, the second one with PCR-negative polychaetes, and the last one with PCR-positive polychaetes. The shrimp were stocked in 500 L fiberglass-tanks containing 300 l of 10 ppt artificial seawater, at 10 shrimp/tank, with adequate aeration. There were two replicates for the first group, one for the second and three for the last group.

Individual, WSSV-inoculated, polychaetes were divided into three equal parts; the head, the middle and the tail parts. A small piece of each part (about 50 mg) were combined for PCR determination; and their status of WSSV infection was identified as very lightly, light, moderate and severe infections. The rest of the polychaete parts (head, middle and tail) were combined and individual polychaetes were immediately frozen at -80°C to ensure infectious state of the virus (Withyachumnarnkul, unpublished data). The shrimp were fed with the frozen polychaetes, which thawed immediately in the aquarium water, at the rate of 3% BW per day. The shrimp were monitored for mortality twice daily for two weeks. At the end of the two weeks, all the survived shrimp were determined for WSSV infection by nested PCR, using the pleopod for crude DNA extraction.

In the third experiment, 80 polychaetes were stocked in a 70 L aquarium, with sand base, in duplicates. The set-up, however, was different from the previous experiment. The sand was placed in the aquarium as a slope and seawater (35 ppt) was stocked in such a way that part of the sand was above and part was below the water level. Five 5-8 g SPF domesticated *P. monodon* were stocked in the aquarium and acclimatized for three days. Then 10 ml of WSSV solution (1:200 dilution) taken from hemolymph of WSSV-infected shrimp was added into the aquarium. After four days, all the shrimp developed WSD and the moribund shrimp were taken for PCR determination of WSSV infection. One week after the last shrimp died, ten new SPF shrimp were stocked in the aquarium and survival rate of the shrimp was monitored twice daily. It was intended that if the shrimp became infected with WSSV, all moribund shrimp would be taken for WSSV nested-PCR. But if the shrimp still survived, they would all be taken for PCR determination and histology examination after one week of rearing. No pellets were provided to the shrimp so that the shrimp would only feed on the polychaetes. Ten polychaetes were then sampled weekly for the PCR determination.

RESULTS

In the first experiment, it was found that 8 out of 12 of wild *P. nuntia* contained WSSV as shown by positive nested PCR reactions, even before WSSV inoculation (Fig. 3). All the positive cases, however, were lightly infected. The proportion of infection was increased to a range of 9/12 to 11/12 (75-90%) after inoculation, up to almost two weeks. Most of the positive cases were very lightly or lightly infected, while some were moderately or severely infected. Three moderate and two severe WSSV infections were found in the first day after the inoculation. But on day 13, all the positive cases were very lightly or lightly ones. The proportion declined dramatically on day 16 and 19, to only 1/12 (8.3%) on day 19 and it was lightly infected. However, the proportion rose to variable levels of 4/12-8/12 (30-70%);

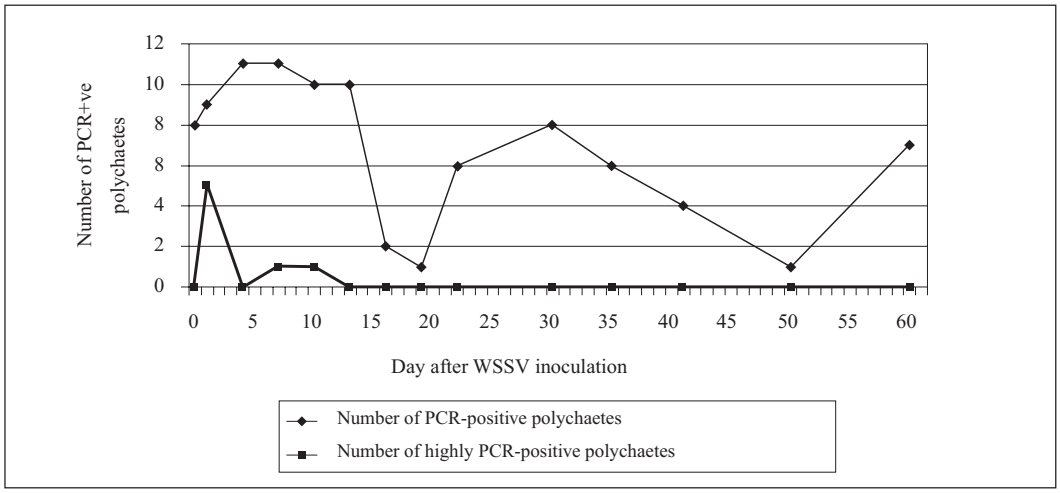

Figure 3. Proportion of wild polychaetes (out of 12) that were detected as positive by nested PCR for WSSV infection following WSSV inoculation. The "positive" polychaetes were those that were positive at the levels of very lightly, light, moderate and severe infection. The "highly positive" were those detectable at moderate and severe infection.

all of the positive cases were very lightly or lightly infected. The proportion dropped to 1/12 on day 50 and rose again to 7/12 on day 60. PCR products from polychaetes with the light PCR reaction revealed sequence specific to that of WSSV.

Histology of *P. nuntia* which were found to be PCR-severely positive revealed no features of WSSV infection, with no hypertrophic nuclei observed (picture not shown).

In the second experiment, when *P. monodon* were fed with the polychaetes, the shrimp survived without any signs of WSD. Survival of shrimp receiving pellets or polychaetes, either negative or positive by PCR, was comparable among the three groups (Fig. 3). At the end of the experiment, all the survived shrimp were PCR-negative.

In the third experiment, all the shrimp injected with WSSV died within five days. They were found to be PCR-positive, and histology also suggested WSSV infection, i.e., hypertrophic nuclei of subcuticular epithelium, gills and other cells of ectodermal and mesodermal origins (Flegel, 2001). Ten new shrimp that were stocked after one more week survived for another one week and they were found to be PCR-negative at the end of the experiment. Histology of the shrimp did not show any features of WSSV infection either (pictures not shown). The polychaetes were found to be variably positive by the PCR (Fig. 5). Proportions of positive animals varied from 3/10 to 9/10 during the 42 day-period in the aquarium; all of them, except one moderate reaction on day 14, were found to be very lightly or lightly infected.

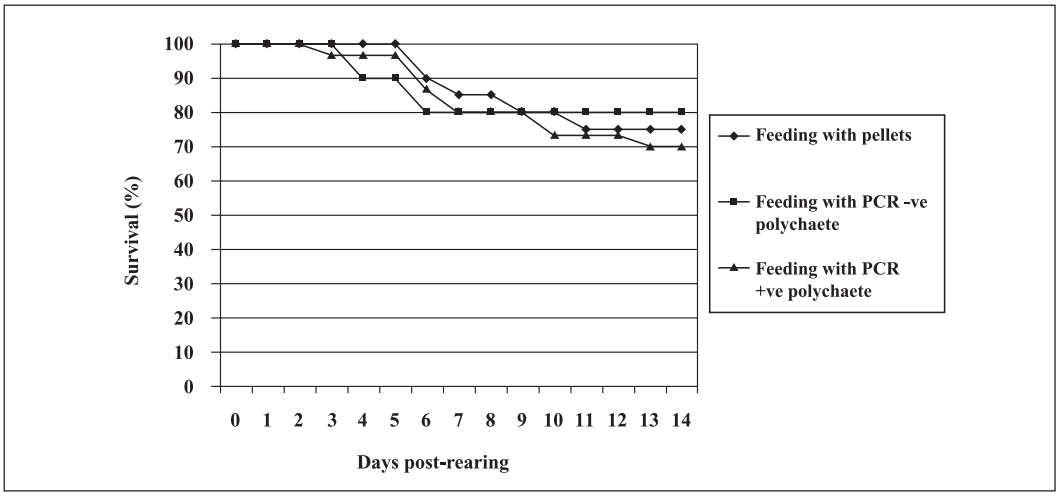

Figure 4. Survival rate of *P. monodon* fed with either commercial pellets, or polychaetes with PCR-negative or polychaetes with PCR-positive for WSSV infection.

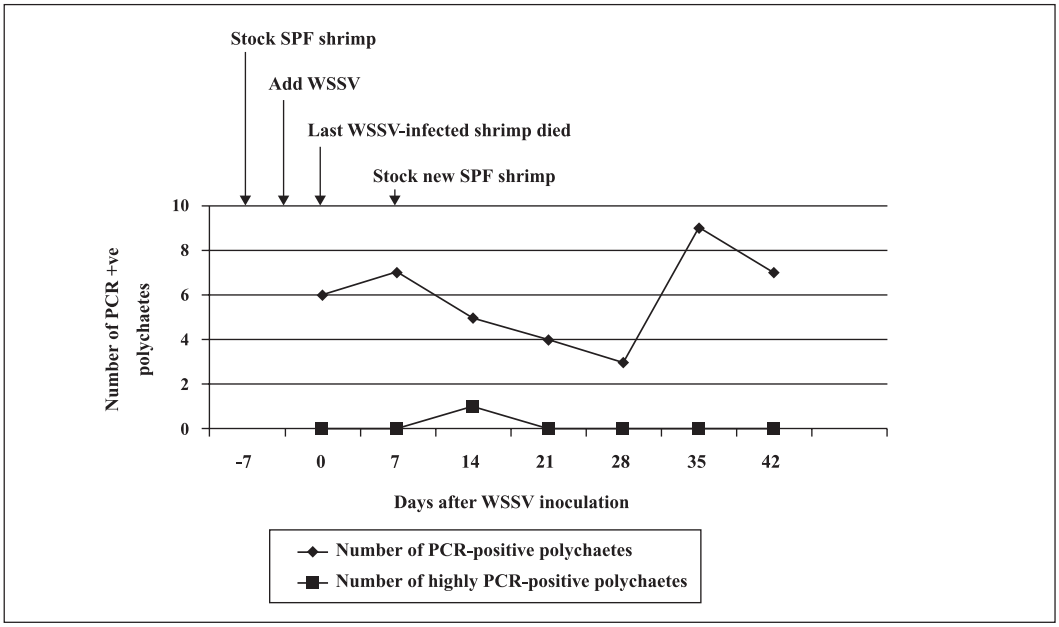

Figure 5. Proportion of wild polychaetes (out of 10) that were detected as positive by nested PCR for WSSV infection following WSSV inoculation and cohabitation with *P. monodon.* The "positive" polychaetes were those that were positive at the levels of very lightly, light, moderate and severe infection. The "highly positive" were those detectable at moderate and severe infection.

DISCUSSION

The findings that *P. monodon* that feed on or in cohabitation with PCR-positive *P. nuntia* did not develop WSD strongly suggest that WSSV cannot be transmitted from *P. nuntia* to *P. monodon*. It was also found that the proportion of WSSV infection in the polychaetes in the first experiment increased following feeding with WSSV-infected meat from *P. monodon*, while in the third experiment, cohabitation with WSSV-infected *P. monodon* did not cause an increase in the proportion. Severity of WSSV infection in the polychaetes in the first experiment was also higher than that in the third experiment. It is possible that feeding the virus to the polychaete might be more effective than immersion in seawater containing WSSV. A possibility that the PCR detected WSSV that remained in the gut lumen of the polychaete but not the virus in the polychaete cells is unlikely as the PCR remained positive after 60 days. WSSV has been found to maintain its infectivity only for a few days in cell-free system (Maeda *et al*., 1997).

Logically, when the polychaetes had WSSV in their bodies and if the virus remained infectious, *P. monodon* that fed on these infected polychaetes should have been infected. The finding that the shrimp were not infected suggested that the virus in the polychaete became non-infectious after a certain period in the polychaete bodies. The presence of the virus or DNA of the virus was confirmed by nested PCR. This finding raises the question whether the nested PCR results were false-positive and if the polychaete had not been infected by WSSV from the beginning. This argument is less likely since the chance of forming the pattern of the bands (Fig. 2) from non-specific amplification should be very low, especially the three band pattern of the severe grading. In addition, the DNA sequence of the PCR product also confirmed the specificity of the detection. Alternatively, it is possible that *P. nuntia* were infected by WSSV, but the virus could not replicate in the polychaete tissue, and/or was attenuated, and became non-virulent in the host. This was also confirmed by an absence of the histological features of WSSV infection in the WSSV-infected polychaete.

It is a puzzle why WSSV stayed in the *P. nuntia* tissues for as long as 60 days, with some fluctuation in the proportion of infection. The viral load was probably low, as most of the PCR-positive cases were in a light or in very lightly reaction levels. Some unknown interactions between the host and the virus might have helped to keep the viral load low. Since PCR detects the DNA of the virus, either dead or live (infectious) particles, it is also possible that the PCR detected the DNA of the virus rather than intact virions.

For practical purposes, the use of *P. nuntia* in shrimp hatcheries should be safe regarding WSSV infection if some precautions are followed. Probably the only procedure needed is to make certain that the polychaetes do not contain infectious WSSV particles in their gut lumens, as wild polychaetes may feed on WSSV-infected shrimp carcasses. Wild polychaetes should be kept in captivity for about one week before use, to excrete WSSV from the gut lumen. However, the best management is to establish polychaete culture in a WSSV-free environment and use WSSV-free polychaetes to feed broodstock.

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