Bioassay evidence for the transmission of WSSV by the harpacticoid copepod *Nitocra* sp.

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Abstract

White Spot Syndrome Virus (WSSV) is now one of the most devastating and virulent viral agents threatening the penaeid shrimp culture industry and has been responsible for serious economic losses for shrimp farms worldwide. One remarkable characteristic of WSSV is its wide reservoir range, which contributes to its wide geographical distribution. Among epizootiological surveys, there is substantial evidence for WSSV-positive copepods found in shrimp farming ponds. Therefore, copepods are suspected to be the vector of WSSV. In the present study, nested-PCR analysis showed positive results in the harpacticoid copepod *Nitocra* sp. exposed to WSSV by virus–phytoplankton adhesion route. Oral route and intramuscular injection were used to test the pathogenicity of WSSV isolated from the WSSV-positive *Nitocra* sp. For the oral route of infection, *Marsupenaeus japonicus* postlarvae were fed with WSSV-positive copepods. The shrimp postlarvae in the infected treatment became WSSV-positive and occurred 52.50 ± 5.00% mortality which was significant higher (*P* < 0.05) than that in the control treatment (20.00 ± 0.00%) when postlarvae were fed with WSSV free copepods. In the intramuscular injection challenge, *M. japonicus* juveniles were injected with the copepods inoculum extracted from the WSSV-positive *Nitocra* sp., and showed 72.50 ± 9.57% mortality which was also significant higher (*P* < 0.05) than that in the control treatment (22.50 ± 5.00%) when juveniles were received mock injection of a tissue homogenate prepared from WSSV-negative *Nitocra* sp. Based on these laboratory challenge studies, it was confirmed that the copepods can serve as a vector in WSSV transmission.

1. Introduction

Though penaeid shrimp farming has undergone rapid development world-wide during last four decades, successful production has been hampered by viral diseases since 1993. Of these viral diseases, White Spot Syndrome (WSS) is one of the most devastating viral diseases affecting most of the commercially cultivated penaeid shrimp species. The first outbreak attributed to the WSS virus was reported in farmed *Marsupenaeus japonicus* in Japan in 1993 (Inouye et al., 1994; Momoyama et al., 1994, 1995; Nakano et al., 1994), and adequate attention since then has been paid to the virus as a result of the large-scale mortality (100% cumulative mortalities can be reached within 3–10 days under farming conditions) and severe economic losses associated with it (Chou et al., 1995; Lightner, 1996; Lightner et al., 1998). WSS in penaeid shrimp is characterized by inclusions or spots on the cuticle, sometimes accompanied by an overall red coloration of the body. The virus was recently assigned to *Whispovirus* (genus) within a new virus family, the *Nimaviridae* (van Hulten et al., 2001; Mayo, 2002; Vlak et al., 2004).

The WSSV has a wide reservoir range, including copepods, shrimp, crayfish, crab, lobster, freshwater crab and prawns. This peculiar characteristic has contributed to its
success over a wide geographic distribution (Huang et al., 1995; Lo et al., 1996a, b, 1997, 1998; Peng et al., 1998; Chou et al., 1998; Maeda et al., 1998; Wang et al., 1998; Chen et al., 2000; Liu et al., 2000; Sahul Hameed et al., 2001, 2002, 2003; Musthag et al., 2006).

Although it has been suspected for over 10 years that copepods are the host or vector of WSSV (Huang et al., 1995; Lo et al., 1996a; He et al., 1999; Liu et al., 2000), presently there is no definitive bioassay evidence that copepods are a vector for WSSV. Therefore, the objective of the present study is to assess the possibility that copepods could serve as a vector in WSSV transmission.

2. Materials and methods

2.1. Test organisms

2.1.1. Harpacticoid copepod Nitocra sp.

The harpacticoid copepod Nitocra sp. was originally collected from penaeid shrimp farming ponds from Qingdao, PR China in 2004. Since then, this species has been cultured in tanks filled with sterile seawater maintained at 20 °C (salinity 32 ppt). The population was fed a daily ration of the two species of algae (Isochrysis zhanjiangensis and Platymonas subcordiformis in the ratio of 3:1) to give a final algal density of 20,000 cells ml⁻¹ in the stock culture tanks.

Prior to the onset of the current study, representative samples of the population were analyzed for WSSV using nested-PCR to ensure they were virus-free (Kong et al., 2003).

2.1.2. Penaeid shrimp

Postlarvae and juveniles of the penaeid shrimp M. japonicus were obtained from a hatchery at Rongcheng, Shangdong Province, PR China. The postlarvae PL 10 (0.002–0.003 g) were maintained in 50-L tanks filled with aerated natural seawater at 23 °C (salinity 32 ppt), and fed three times daily with WSSV-negative Artemia nauplii.

The juvenile shrimps (1.8–2 g) were kept in two 300-L fiber glass tanks in the laboratory and fed ad libitum twice a day with a commercial pellet feed at a rate of 5% body weight day⁻¹.

Prior to the experiments, the shrimps were acclimatized to 25 ± 2 °C, and were randomly selected and diagnosed WSSV free by nested-PCR using the primers designed by Kong et al. (2003).

2.2. Preparation of WSSV inoculum

The WSSV inoculum was prepared as described previously (Vidal et al., 2001). In brief, soft tissue (400 g) from the cephalothorax and muscle of naturally WSSV-infected Fenneropenaeus chinensis was homogenized in 0.9% NaCl (1 g tissue/10 ml 0.9% NaCl). The homogenate was centrifuged at 6000g for 15 min at 4 °C, and its supernatant was re-centrifuged at 10,000g for 20 min at 4 °C before the final supernatant was filtered through a 0.2-μm membrane filter. Aliquots were transferred to 50-ml plastic centrifuge tubes and then stored at −80 °C. Before storage, the presence of WSSV in the tissue sample and the final supernatant fluid was determined by a nested-PCR assay (Kong et al., 2003).

2.3. Preparation of harpacticoid copepod Nitocra sp. inoculum

Approximately 200 WSSV-positive or negative harpacticoid copepods were filtered through a 100-μm screen and washed thoroughly with 0.9% NaCl before being transferred to 1.5-ml microfuge tubes. The inoculum was prepared as previously described (Vidal et al., 2001).

2.4. Infectivity experiment

2.4.1. Challenge of WSSV with the harpacticoid copepod Nitocra sp.

The first challenge study was the infectivity experiment of WSSV in harpacticoid copepod Nitocra sp., which comprised of two experimental treatments: test and negative control (10 replicates each). In each replicate, copepods (1 individual 10 ml⁻¹) were kept in a 20-L aquarium containing sterile seawater. The quaria of each treatment were kept separated in two illuminated incubators (20 °C, 12-h photoperiod) in order to prevent cross-contamination.

In the test treatment, copepods were exposed to WSSV by the virus–phytoplankton adhesion route, described previously by Zhang et al. (2006). In short, a mixed algal solution of I. zhanjiangensis (1500 ml) and P. subcordiformis (500 ml) were first mixed with 300 ml of the viral inoculum for 0.5-h, and then divided into 10 aliquots to feed to the copepods in each replicate aquarium. The copepods were fed daily at 08:00 until all challenge experiments were completed. On Day 6, 10–15 animals from each replicate aquarium of the test treatment were filtered through a 100-μm screen, and rinsed three times with sterile seawater before transferred to a fresh 50-ml beaker containing 40 ml of sterile seawater. The animals were left to starve for 24 h before assayed for WSSV infection.

The control copepods were treated in the same manner as the test treatment, except the algal feeding solution was first mixed with 0.9% NaCl instead of the WSSV inoculum.

2.4.2. Infection of M. japonicus postlarvae via the oral route with the WSSV-positive harpacticoid copepod Nitocra sp.

Infection of M. japonicus postlarvae by WSSV-positive harpacticoid copepod Nitocra sp. prey consisted of four treatments: two negative controls (NCO, NSO), one positive control (PSO) and one infected treatment (PCO). Each treatment was carried out in four replicates. In each replicate, 10 M. japonicus postlarvae were individually acclimated in a 1-L beaker with 900 ml sterile seawater. The
rearing seawater was changed 50% daily before the feeding. Replicates of each treatment were kept separated in four illuminated incubators (25°C, 12-h photoperiod) in order to prevent the cross-contamination. The shrimp postlarvae were fed twice a day at 08:00 and 18:00. The experiment lasted 15 days, upon which the postlarvae were left to starve for 24 h before sampling.

In the negative control treatment NCO (NCO treatment), the postlarvae were fed with WSSV-negative copepods at a density of 1 individual per 2 ml. In the PCO treatment, the postlarvae were treated in the same manner as the organisms in the NCO treatment, except that they were fed with WSSV-positive copepods. To prevent the accidental transfer of WSSV from the medium in which the WSSV-positive copepods were reared, the copepods were filtered through a 100-μm screen, rinsed three times on the screen with sterile seawater, and starved for 24 h in sterile seawater.

In the negative control NSO treatment the shrimp were fed with minced meat from WSSV-negative *F. chinensis* at a rate of 5% body weight day⁻¹. The shrimp serving as positive control (PSO treatment) were fed with minced meat from WSSV-infected *F. chinensis* at the same rate and frequency as the NSO treatment. The postlarvae were examined twice a day before the feeding. Dead postlarvae were removed when observed and cumulative mortality was calculated. Mortalities were analyzed using the Analysis of Variance (ANOVA) following software SPSS 11.0.

### 2.4.3. Infection of *M. japonicus* juveniles via the intramuscular injection with WSSV-positive harpacticoid copepod *Nitocra* sp. inoculum

In the third challenge study, the juveniles (*M. japonicus*) were infected with WSSV by an intramuscular injection (*Yan et al., 2007*). The challenge study comprised of four experimental treatments: PCI treatment served as the test treatment, whereas NCI treatment and NNI treatment were the negative controls, and PSI treatment served as the positive control (each consisting of four replicates). In each replicate, 10 juvenile shrimp were kept in a 50-L fiber glass tank. The experimental shrimp of PCI treatment were inoculated intramuscularly in the dorso–lateral aspect of the fourth abdominal segment with an inoculum (50 μl per animal) prepared from the WSSV-positive harpacticoid copepods, whereas shrimp in PSI treatment were injected with a WSSV inoculum (50 μl per animal). The WSSV inoculum was diluted 1:100 with 0.9% NaCl prior to inoculation. Shrimps in the NCI and NNI treatments received mock injections of inoculum prepared from WSSV-negative harpacticoid copepods and 0.9% NaCl, respectively. All of the shrimp were fed twice a day at 08:00 and 18:00 with a commercial pellet feed at a rate of 5% body weight day⁻¹. The rearing seawater was changed 70% daily before the morning feeding.

The animals were examined twice a day before the feeding. Dead postlarvae were removed when observed. Cumulative mortality was calculated and analyzed as described above.

### 2.5. Molecular diagnosis of WSSV infection

The two sets of primer sequences used in the present work were previously reported by *Kong et al. (2003)*. The expected size of the amplified fragment was 1221 bp for the one-step PCR reaction and 982 bp for the nested-PCR reaction. The PCR amplification for the one-step and nested-PCR reactions was carried out in a 25 μl reaction mixture containing 1 μl template DNA, 2.5 μl 10×*Taq* buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1.5 mM MgCl₂, 15 mM each primer, 200 mM of dATP, dGTP, dCTP and dTTP, 2.5 U* Taq* polymerase (Promega, Shanghai) and sterile double-distilled de-ionized water to make up the final volume. Amplification was carried out in a Mastercyler thermocycler (Eppendorf, Germany), with an initial denaturation step at 94°C for 5 min and 30 cycles of 94°C 40 s, 58°C 40 s, 72°C 2 min, and a final extension at 72°C for 10 min. Following this, an aliquot of the PCR product was analyzed by 1.0% agarose gel electrophoresis stained with ethidium bromide. The gel was then visualized under UV light and photographed.

Samples that tested negative by one-step PCR were subjected to the nested-PCR using 1 μl of the one-step amplified product as a DNA template.

### 2.6. DNA extraction

Template DNA for the PCR assays was prepared by extracting DNA from the harpacticoid copepod *Nitocra* sp. (10–15 live ones), *M. japonicus* postlarvae (three dead ones) and gill tissues of juvenile shrimps (two dead ones) following the method described by *Wang et al. (2000)*. Materials were fixed in SEMP–Tris (10 mM Tris–HCl, 70 mM EDTA, 1% SDS, 0.5% mercaptoethanol, phenol saturated, pH 8.0), boiled and extracted via ethanol precipitation. Dried DNA was dissolved with Tris–ethylenediaminetetraacetic acid (TE) (pH 8.0) buffer.

### 3. Results

#### 3.1. Detection of WSSV in experimentally infected harpacticoid copepod *Nitocra* sp.

Nested-PCR was positive only for the animals exposed to WSSV by the virus–phytoplankton adhesion route. No positive results occurred in the control treatment (Fig. 1). One-step PCR analysis could not detected WSSV DNA in the copepods.

#### 3.2. Infectivity of *M. japonicus* postlarvae exposed to WSSV when fed with WSSV-positive harpacticoid copepod *Nitocra* sp.

The WSSV caused 52.50 ± 5% mortality in the test treatment (PCO) in which *M. japonicus* postlarvae were fed with WSSV-positive harpacticoid copepods in the
The experimental period of 15 days (Fig. 2). The cumulative mortality in the negative control treatment (NCO) was only 10.00 ± 14.14% due to artificial operation or natural mortality when the postlarvae were fed with WSSV-negative copepods. The cumulative mortality in the PCO treatment was significantly higher than that of the NCO treatment ($P < 0.05$). The WSSV caused 75.00 ± 12.91% mortality in the positive control (PSO) treatment when the animals were exposed to minced meat of WSSV-infected *F. chinensis*. The mortality in the negative control (NSO) treatment was 20.00 ± 0% when the postlarvae were fed with minced meat of WSSV-negative shrimp. There was a significant difference between the mortalities of the PSO and NSO treatments ($P < 0.05$).

The samples from the PCO and PSO treatments tested positive for the presence of WSSV DNA by nested-PCR, whereas all samples from the NCO and NSO treatments were negative (Fig. 3). Of the samples from the PSO treatment, one was one-step PCR positive while three were nested-PCR positive. All samples from the PCO treatment were nested-PCR positive.

3.3. Infectivity of *M. japonicus* juveniles exposed to WSSV via the intramuscular injection with inoculum prepared from the WSSV-positive harpacticoid copepod *Nitocra* sp.

The WSSV caused 72.50 ± 9.57% mortality in the PCI treatment in which *M. japonicus* juveniles were injected with an inoculum prepared from the WSSV-positive harpacticoid copepod *Nitocra* sp. during the 20-day experimental period (Fig. 4). The percent mortality in the NCI treatment was 22.50 ± 5.00% when the juveniles received mock injection of the inoculum prepared from WSSV-negative *Nitocra* sp. The cumulative mortality in the PCI treatment was significantly higher than that of the NCI.
In the first challenge study, harpacticoid copepod was successfully infected with the WSSV using the virus–phytoplankton adhesion route (Fig. 1). These results confirmed the finding that phytoplankton can carry WSSV that have dissociated from infected animals. This implies that filter feeders, especially zooplankton, can accumulate WSSV when ingesting phytoplankton carrying WSSV particles. The results of this study indicated that the filter feeding mode of nutrition of harpacticoid copepods is responsible for facilitating WSSV transmission in these animals (Fig. 1). Similar results were obtained for rotifers (Zhang et al., 2006).

In 1994, Huang et al. (1995) carried out an epizootiological survey from May to July in shrimp ponds in Shandong and Liaoning provinces (PR China) where WSSV first appeared in 1993. Their survey indicated that WSSV prevalence was higher in live copepods (60%) than in shrimps (52%) using an ELISA test. Furthermore, the virus appeared in live copepods 20–40 days earlier.

Similar results were also obtained in our epizootiological survey from May to October in 2003 in three shrimp ponds that were plagued by the WSSV since 1993. Of 48 copepod specimens sampled from these ponds on May 14, 18 individuals (37.5%) tested WSSV-positive using the PCR-dot-blot hybridization method. After the first sample period, the shrimp postlarvae were kept in ponds until the end of May. Since this time, samples were taken every month to survey the prevalence of WSSV. The survey results showed that the WSSV prevalence in copepods was over 60% from June to October. The highest prevalence (85.7%) was detected in August after WSS occurred.

In addition, previous work found the receptor for WSSV on the cell membrane of the calanoid copepod Acartia clausi, providing further evidence that the copepod is the WSSV host (Feng et al., 2005).

In the present study, WSSV-positive harpacticoid copepods which were exposed to WSSV by the virus–phytoplankton adhesion route caused high mortalities in M. japonicus postlarvae and juveniles when exposed to the virus through the oral route and intramuscular injection, respectively (Figs. 2 and 4). These mortalities support our hypothesis that there is a correlation of WSS and its suspect vector—copepods.

It has been well demonstrated that copepods serve vector to the causative agent of cholera, Vibrio cholerae (Huq and Colwell, 1996). The studies of Sochard et al. (1979) and Huq et al. (1983) indicated that copepods carry V. cholerae on their body surface, in their gut and on their egg sac surface, and V. cholerae survival can be enhanced when live copepods are present. Furthermore, it is well recognized that outbreaks of epidemic cholera and copepod blooms in the natural aquatic environment are correlated. Compared with the findings of the association of V. cholerae with copepods, more evidence is required to establish the correlation between the incidence of WSS and the presence of copepods in shrimp rearing ponds.
In summary, the results of this study demonstrated that copepods can serve as a vector in the WSSV transmission via the oral route. Further work is needed to determine whether WSSV can propagate between copepod species.

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References


