Can white spot syndrome virus be transmitted through the phytoplankton→rotifer→artemia→shrimp pathway?

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The transmission of white spot syndrome virus (WSSV) in the aquatic environment by the pathway of phytoplankton through rotifer to artemia and shrimp was investigated. The phytoplankton Alexandrium tamarense and Alexandrium minutum were co-cultured with adult Fenneropenaeus chinensis infected with WSSV and were assayed by whole cell fluorescence in situ hybridization (WFISH) with probe specific for WSSV labeled with 5-carboxyfluorescein at 5'-end to study whether they could carry WSSV. Then, the WSSV positive phytoplankton was exposed to the rotifer Brachionus urceus and was assayed by dot blot hybridization with digoxigenin labeled DNA probe. Further experiments were conducted to feed artemia Artemia franciscana with the WSSV positive rotifers and feed juvenile shrimps F. chinensis. Our results showed that the pytoplankton were WSSV-positive after 24 h incubation. The dot-blot diagnosis revealed WSSV-positive results in the rotifers exposed to WSSV positive phytoplankton. The cumulative mortality of shrimp and dot blot diagnosis showed that the shrimp can be infected by the food chain of phytoplankton→rotifer→artemia→shrimp.

Key words: White spot syndrome virus (WSSV), whole cell fluorescence in situ hybridization (WFISH), Alexandrium tamarense, Alexandrium minutum, Brachionus urceus, Artemia franciscana, Fenneropenaeus chinensis.

INTRODUCTION

Shrimp viral diseases have become a major impediment to commercial shrimp farming worldwide (Lightner, 1998). White spot syndrome virus (WSSV), a large double-stranded circular DNA virus, that is now assigned to the virus family Nimaviridae, genus Whispovirus (Van Hulten et al., 2001; Mayo, 2002; Vlak et al., 2002), is the most virulent virus reported in the farmed shrimps (Flegel and Alday-Sanz, 1998; van Hulten et al., 2001) and has become a major deterrent in the growth and sustainability of shrimp aquaculture (Lightner, 1996). The wide host range of this virus includes many aquatic crustaceans (Lo et al., 1996a, b; Lo et al., 1997; Peng et al., 1998; Otta et al., 1999; Wang et al., 1998; Rajendran et al., 1999; Hossain et al., 2001). The main infected pathway of WSSV is by food and hosts of WSSV were considered as the infectious source of WSSV.

Phytoplankton plays important roles in micro-ecology of shrimp ponds. Most importantly, shrimps of different developmental stages feed on phytoplankton directly or indirectly in the shrimp pond (Gómez-Aguirre and Martínez-Co’rdova, 1998). So, the horizontal pathway of WSSV transmission has been the main focus of recent researches. Some studies showed that marine microalgae could carry WSSV and the WSSV-positive microalgae could infect rotifer and shrimp (Zhang et al., 2006; Liu et al., 2007). But the transmission of WSSV by the food chain is still to be elucidated. In this study, we investigated the transmission of WSSV through the food chain of phytoplankton→rotifer→artemia→shrimp.

MATERIALS AND METHODS

Preparation of crude WSSV extracts

Fifty grams of the muscle of Fenneropenaeus chinensis with severe WSSV infection, from Haiyang Yellow Sea Shrimp Farm in Yantai China, were added into 200 ml Penaeid Physiological Buffer (PPB)

Abbreviations: WSSV, White spot syndrome virus; WFISH, whole cell fluorescence in situ hybridization.
Specificity tests showed that there was no cross reaction with Sangon Biological Engineering Technology and Service Co., Ltd) with fluorescent dye 5-carboxyfluorescein (FAM) at 5'end (Shanghai WSSV genomic sequence (GenBank No. U50923), starting from experimental procedure, the rotifers were fasted and there were no infectious hypodermal and hematopoietic necrosis virus (IHHNV), and hepatopancreatic parvovirus (HPV) (Durand et al., 2003).

The probe, 5'-AGC CAT GAA GAA TGC CGT CTA TCA CACA- 3', the probe for fluorescence in situ hybridization (WFISH) which indicates the distribution of WSSV in the phytoplankton properly. The procedure is as follows: the phytoplankton cells were washed three times in 5 × PBS (684 mM NaCl, 14 mM KCl, 405 mM Na2HPO4, 12H2O and 7.5 mM KH2PO4; pH7.5) at 2000 ×g for 5 min. The supernatant was discarded and the precipitum was used to feed starved rotifers.

The WSSV positive phytoplankton were collected and washed three times with sterile seawater at 2000 ×g for 5 min. The supernatant was discarded and the precipitum was used to feed starved rotifers. 

Adult F. chinensis with average length of 14.5 to 15.0 cm were purchased from Nanshan Market, Qingdao (Shandong Province, China) during May 2008. Juvenile F. chinensis (average body length, 0.85 to 1.00 cm) used in this experiment were obtained from Aquaculture Institute of Rizhao (Shandong Province, P. R. China). Adult and juvenile shrimps were maintained in 70 L tanks, and fed with WSSV-free commercial dry diet daily. Unconsumed food and feces were removed carefully with siphons.

Preparation of probe for fluorescence in situ hybridization

The probe, 5'-AGC CAT GAA GAA TGC CGT CTA TCA CACA- 3', for the use of dot-blot hybridization kit to avoid the interruption of autofluorescence of the phytoplankton, because the autofluorescence of the phytoplankton in the digestive canal of zooplankton can not be removed properly. The procedure is as follows: the phytoplankton cells were washed three times in 5 × PBS (684 mM NaCl, 14 mM KCl, 405 mM Na2HPO4, 12H2O and 7.5 mM KH2PO4; pH7.5) at 2000 ×g for 5 min. The supernatant was discarded and the precipitum was used to feed starved rotifers.

Transmission of WSSV from WSSV positive phytoplankton to rotifers

The WSSV positive phytoplankton were collected and washed three times with sterile seawater at 2000 ×g for 5 min. The supernatant was discarded and the precipitum was used to feed starved rotifers. After sampling, B. urceus was fed with Chlorella sp. (about 10⁶ cells ml⁻¹).
ad libitum twice a day, and 30% of the rearing seawater was changed daily. The control rotifers were treated in the same manner as the test organisms, except that A. tamarense and A. minutum were WSSV free.

Transmission of WSSV from WSSV positive rotifers to artemia

Transmission of WSSV from WSSV positive rotifers to artemia also included a test and control treatment, each being carried out in 10 replicates. Rotifer B. urceu were filtered through a 75-µm screen, and then rinsed three times on the screen with sterile seawater before being fed to the A. franciscana. A. franciscana (80 to 100 per aquarium, body length of about 1.5 to 2.0 mm) were acclimated individually in 250 ml beaker with 200 ml sterile seawater. Replicates of each treatment were kept separately in two illuminated incubators (20°C, 12-h photoperiod) in order to prevent the cross-contamination. In the infection treatment, the artemia were fed with WSSV-positive rotifers at a density of 20 ind. ml−1. In the control treatment, the artemia were fed with WSSV-negative rotifers at the same density and frequency as the infection treatment. The artemia were examined under microscope at 2 h interval. When the rotifers were found in the digestive canal of the artemia, the artemia were collected, washed with fresh sterile seawater and placed in the fresh sterile seawater and starved to empty the digestive canal. The WSSV of the A. franciscana artemia was also detected by dot-blot hybridization.

Infection of juvenile F. chinensis with WSSV positive artemia

Before the experiment, the juvenile F. chinensis were detected as WSSV free by dot blot and were starved for 24 h. The test treatments were carried out in 5 replicates with 50 shrimps in each group. The procedure of infection of juvenile F. chinensis with WSSV-positive artemia was similar to that of “2.6. Transmission of WSSV from WSSV positive rotifers to artemia” except that the artemia were filtered through a 100-µm screen, and then rinsed three times on the screen with sterile seawater before being fed to the shrimps. The shrimps were fed with WSSV-positive artemia twice on the first day with a density of 20 ind.ml−1. The digest canal of the juvenile F. chinensis was examined for the presence of artemia under the microscope. When artemia were found in the digestive canal of the juvenile F. chinensis, the shrimp were fed with artificial diet twice a day to the end of the experiment. Unconsumed food and feces were removed carefully with siphons. The WSSV of the shrimp was diagnosed by dot-blot once a day and dead shrimps were removed and cumulative mortality levels were calculated. Mortalities were analyzed by ANOVA in software Excel.

RESULTS AND DISCUSSION

Phytoplankton is the base of the food web in pond cultures. In this study, the results of WFISH showed that both A. tamarense and A. minutum were WSSV positive after 24 h of co-culturing with WSSV-infected adult shrimp F. chinensis (Figure 1). Several microalgae have been reported as carriers of WSSV (Liu et al., 2007; Zhang et al., 2006) but they are thought not to be the “true” host for the WSSV. It was suggested that the virus was just briefly (for <10 days) associated (they become passive carriers) with the microalgae tested (Liu et al., 2007). The taxonomy of microalgal viruses is believed to be very different from that of WSSV that do not infect phytoplankton from a genetic perspective (Vlak et al., 2002; Chen and Suttle, 1996).

The WSSV positive phytoplankton A. tamarense and A. minutum were both used to feed rotifers B. urceu. Two hours later, both phytoplankton A. tamarense and A. minutum were found in the rotifers’ digestive canal under the microscope. The rotifers were diagnosed WSSV positive by dot blot hybridization after 12 h of exposing the WSSV positive phytoplankton to the rotifers. There was no difference in the infectivity between WSSV positive A. tamarense and A. minutum. Both infected the rotifers at the same time. This implies that filter feeders, especially zooplankton, ingest phytoplankton that carried WSSV, and therefore accumulate viral particles within certain time. The results revealed that the filter feeding habit of the animals might be responsible for making rotifer WSSV-positive (Table 1). Yan et al. (2007) reported that cell membranes from the rotifer B. urceu specifically bind WSSV, suggesting that rotifer is a potential host of WSSV.

Whether artemia is the host or vector of WSSV has been debated for over 10 years (Huang et al., 1995; Lo et al., 1996a; He et al., 1999; Liu et al., 2000; Hameed et al., 2002). Hameed et al. (2002) mixed viral suspension with rice bran and fed artemia with the mixture and found that the artemia did not carry WSSV. However, in this study, the rotifers B. urceu were found in the digestive canal of artemia A. franciscana after 2 h of exposing the WSSV positive rotifers B. urceu to the artemia and the artemia were diagnosed WSSV positive by dot blot hybridization 24 h later (Table 1). These results implied that the rotifers might be WSSV carriers with high WSSV transmissibility. The transmissibility of WSSV in the rice bran may be reduced or eliminated in the experiments of Hameed et al. (2002).

After 2 h of being fed with the WSSV positive artemia, the artemia was found in the digestive canal of juvenile F. chinensis and 24 h later the shrimps were diagnosed WSSV positive by dot blot hybridization. All the shrimps in the treatment group died, whereas the control group’s survival rate was 80% within 136 h (Figure 2). All shrimps in the control group in this study were diagnosed WSSV negative by dot blot hybridization.

In conclusion, the results of the study showed that WSSV is transmitted through the food chain of phytoplankton→rotifer→artemia→shrimp. However, further experiments are still needed to develop a cell culture system from artemia A. franciscana to study the gene function, WSSV replication and host–virus interactions.

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Figure 1. Diagnosis of WSSV in the phytoplankton by whole cell fluorescence *in situ* hybridization (WFISH). (A) and (E) are micrographs of *A. minutum* and *A. tamarense* incubated with 24 h after incubation with WSSV-infected
Table 1. The WSSV diagnosis of the food chain of phytoplankton, rotifer, artemia, shrimp.

<table>
<thead>
<tr>
<th>Tested species</th>
<th>Diagnostic result of WSSV</th>
<th>Prevalence (%)</th>
<th>Initial time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoplankton (Alexandrium tamarense and A. minutum)</td>
<td>+</td>
<td>2/2 (100%)</td>
<td>24</td>
</tr>
<tr>
<td>Rotifer (Brachionus urceus)</td>
<td>+</td>
<td>10/10 (100%)</td>
<td>12</td>
</tr>
<tr>
<td>Artemia (Artemia franciscana)</td>
<td>+</td>
<td>10/10 (100%)</td>
<td>24</td>
</tr>
<tr>
<td>Shrimp (Fenneropenaeus chinensis)</td>
<td>+</td>
<td>5/5 (100%)</td>
<td>24</td>
</tr>
</tbody>
</table>

Figure 2. Survival ratio of WSSV-infected juvenile *F. chinensis* after being fed with WSSV positive *Artemia franciscana*. Fifty samples used in each group were carried out in 5 replicates.

REFERENCES


Lo CF, Ho CH, Chen CH, Liu KF, Chiu YL, Yeh PY, Peng SE, Hsu HC, Liu HC, Chang CF, Su MS, Wang CH, Kou GH (1997). Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Peneaus monodon* with a special emphasis on