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Full Length Research Paper

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-carboxyfluoroscein at 5'-end to study whether they could carry WSSV. Then, the WSSV positive phytoplankton was exposed to the rotifer *Brachionus urceu* 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* with the artemia. 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.

Abbreviations: WSSV, White spot syndrome virus; **WFISH,** whole cell fluorescence *in situ* hybridization.

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 (Go'mez-Aguirre and Martýnez-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)

(Huang et al., 1999) and homogenized at 3000 rpm for 5 min at 0°C. Then, the mixture was centrifuged at 5520 ×g for 30 min at 4°C and the supernatant was centrifuged at 9820 ×g for 30 min at 4°C. This was repeated twice. The supernatant was centrifuged at 15300 ×g for 60 min at 4°C, and the precipitum was resuspended in PPB and stored at -20°C before use.

Phytoplankton, rotifer, artimia and shrimp species

Dinoflagellate *Alexandrium tamarense* and *Alexandrium minutum* were cultured in our laboratory in enriched natural seawater medium f/2 and were incubated at 20°C with a light intensity of 2000 lx at the surface of the cultures, and only phytoplankton in the exponential growth phase were used.

The rotifer *Brachionus urceu* was originally hatched from sediment samples of a shrimp-rearing pond collected in 2008 from Rushan, China. After hatching, the population was cultured in 40-L aquaria filled with sterilized seawater (salinity 32 ppt) in an illuminated incubator (20°C, 12-h photoperiod). The zooplankton fed on marine unicellular microalgae *Chlorella* sp. twice a day at a density of 1.2×10^5 cells ml^{-1}. The microalgae *Chlorella* sp. in the digestive canal of the rotifers was detected with a microscope (Olympus BH-2, Japan) with magnification of ×400. Before the experimental procedure, the rotifers were fasted and there were no food detected in the digestive canal of the rotifers.

Artemia franciscana cysts (Unibest™ 020730, marine strain) were decapsulated and subsequently incubated for 24 h in 6 L cylindrical tanks with water at 20°C and salinity at 32 ppt, strong bottom aeration (near saturated oxygen levels, >5 ppm) and light (2000 lx) at a maximum density of 2 $g.L^{-1}$. The number of newly hatched *A. franciscana* artemia obtained was estimated by sub-sampling (ten replicates of 1 ml subsamples). *A. franciscana* artemia were then stocked at a density of 50 artemia.ml^{-1} in 5 L cylindrical tanks with strong bottom aeration (near saturated oxygen levels, >5 ppm) and light (2000 lx), and enriched with 0.2 $g.L^{-1}$ of AlgaMac 2000® as recommended by the manufacturer (Aquafauna Bio-Marine Inc.).

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', used for the detection of WSSV, was selected from a region of WSSV genomic sequence (GenBank No. U50923), starting from nucleotide 1032 to nucleotide 1060. It was synthesized and labeled with fluorescent dye 5-carboxyfluoroscein (FAM) at 5'end (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd). Specificity tests showed that there was no cross reaction with shrimp DNA, other baculoviruses DNA such as *Baculovirus penaei* (BP), *Monodon baculovirus* (MBV) and parvoviruses DNA such as infectious hypodermal and hematopoietic necrosis virus (IHHNV), and hepatopancreatic parvovirus (HPV) (Durand et al., 2003).

Interaction of phytoplankton with WSSV

The crude WSSV extracts were diluted 10 times with PPB and filtered through 0.22 µm membrane filters. Then 0.1 ml of diluted virus solution was injected into each adult *F. chinensis* in the lateral area of the fourth abdominal segment with a sterile syringe. Infected adult shrimps were maintained in 2-L aquaria (one adult shrimp per aquarium). Stock cultures of *A. tamarense* and *A. minutum* adjusted to a target concentration of about 10^4 cells ml^{-1} were added to the aquaria two days after acclimation to the test environment. Aquaria were maintained at 20°C and submitted to 12-h photoperiod with a light intensity of 2000 lx at the surface of cultures. The control was treated in the same manner except that the adult *F. chinensis* were not injected with WSSV solution. Twenty-five milliliters of experimental microalgal stock culture was sampled daily to determine whether it contained WSSV by whole cell 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 $Na_2HPO_4.12H_2O$ and 7.5 mM KH_2PO_4; pH7.5) at 2000 ×g for 5 min. The supernatant was discarded and the precipitum was resuspended and fixed in 5 × PBS containing 4% paraformaldehyde for 20 min at room temperature. After centrifugation (1000 ×g for 1 min), cells were dehydrated and diminished with autofluorescence by a serial ethanol solution of 50 and 80% and then dried quickly using an aspirator. The cells were then incubated with CTAB solution (0.8M NaCl, 50 mM EDTA, 0.1% SDS, 1% cetyltrimethylammonium bromide) at 65°C for 8 min. After another centrifugation (1000 ×g for 1 min), cells were resuspended in 200 µl of hybridization buffer (775 mM NaCl, 70 mM sodium citrate at pH 7.0, 5 mM EDTA, 0.01% SDS, 0.1%CTAB) containing 200 ng probe and incubated at 95°C for 3 min to denature genomic DNA. The solution was incubated on ice for 3 min and the cells were then incubated with the probe at 50°C for 1 h. The cells were then washed twice with 5×SSC (750 mM NaCl, 75 mM sodium citate, pH 7.0) at 50°C for 10 min (Adachi et al., 1996). Cells were transferred to slides and viewed under Axioplan fluorescence microscope (1000 or 400× magnification) (Zeiss, Jena, Germany) equipped with a 50 W high pressure mercury bulb and filter combinations (G-450 to 490 nm, FT-510 nm, LP-520 nm). Color photomicrographs were developed on Kodak Ektachrome 1600 (Rochester, NY, USA).

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. The infectivity experiment of WSSV in rotifers comprised of two treatments: test and negative control. Each treatment consisted of 10 replicates. In each replicate, rotifers (5 ind. ml^{-1}) were stocked in sterile 1-L aquaria containing 800 ml of sterilized seawater. Aquaria of each treatment were kept separately in two illuminated incubators (20°C, 12-h photoperiod with a light intensity of 2000 lx at the surface of cultures) in order to prevent cross-contamination.

In the test treatment, starved rotifers were fed WSSV positive *A. tamarense* and *A. minutum* (about 10^5 cells/ml), the rotifers were examined under microscope at 2 h interval. When the phytoplankton was found in the rotifers' digestive canal, the rotifers were collected, washed with fresh sterile seawater and placed in the fresh sterile seawater and starved to empty the digestive canal. Then, the WSSV in the rotifers was diagnosed with a dot-blot hybridization kit to avoid the interruption of autofluorecence of the phytoplankton, because the autofluorecence of the phytoplankton in the digestive canal of zooplankton can not be removed properly. The dot-blot hybridization kit was purchased from the Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, utilizing a digoxigenin labeled DNA probe. After sampling, *B. urceus* was fed with *Chlorella* sp. (about 10^5 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. urceus* 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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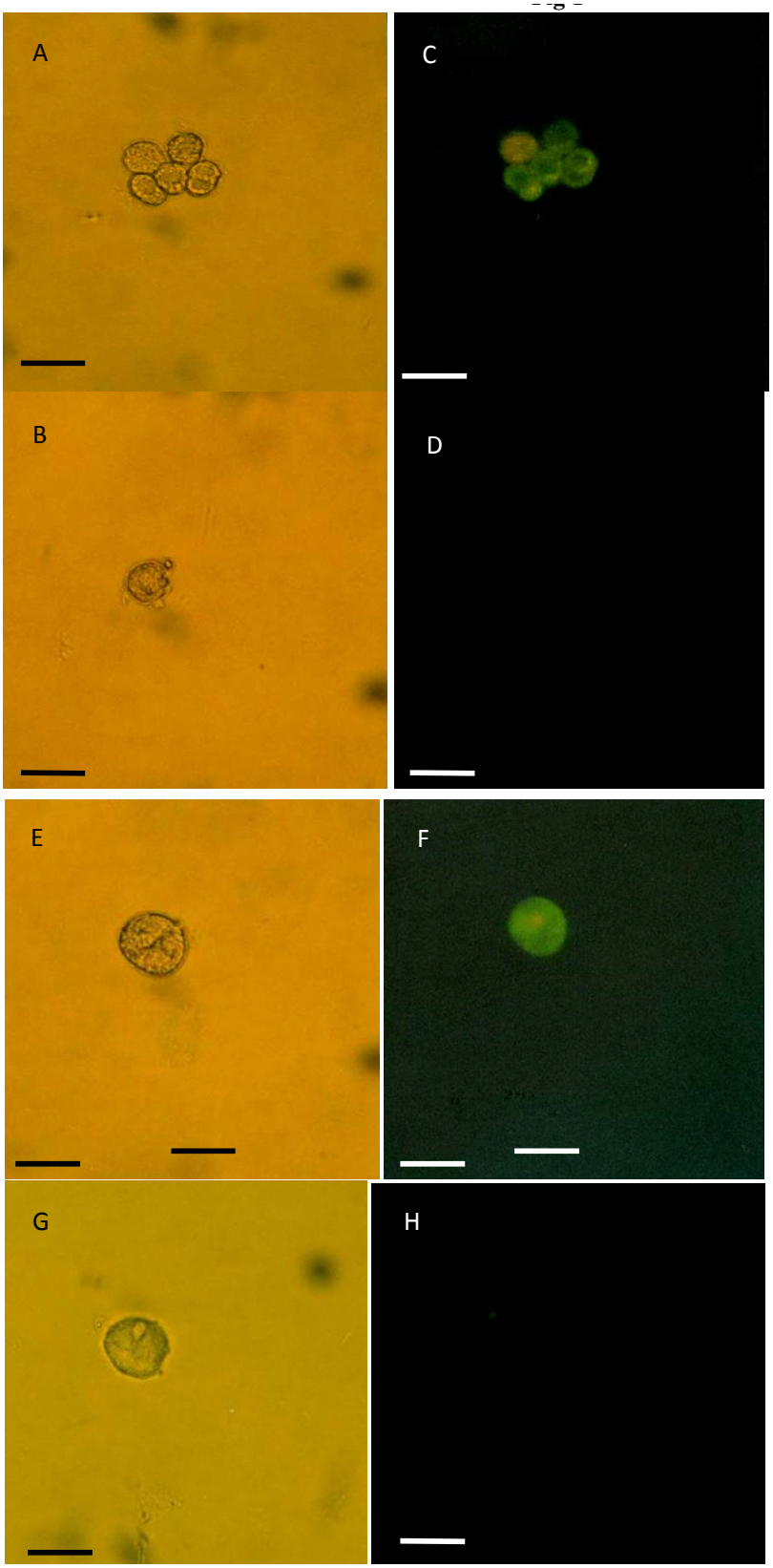

Figure 1. Diagnosis of WSSV in the phytoplankton by whole cell fluorescence *in situ* hybridization (WFISH). (A) and (E) are micrographs of *A. minutum* and *A. tamarense* incubated with 24 h after incubation with WSSV-infected

Table 1. The WSSV diagnosis of the food chain of phytoplankton → rotifer → artemia → shrimp.

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

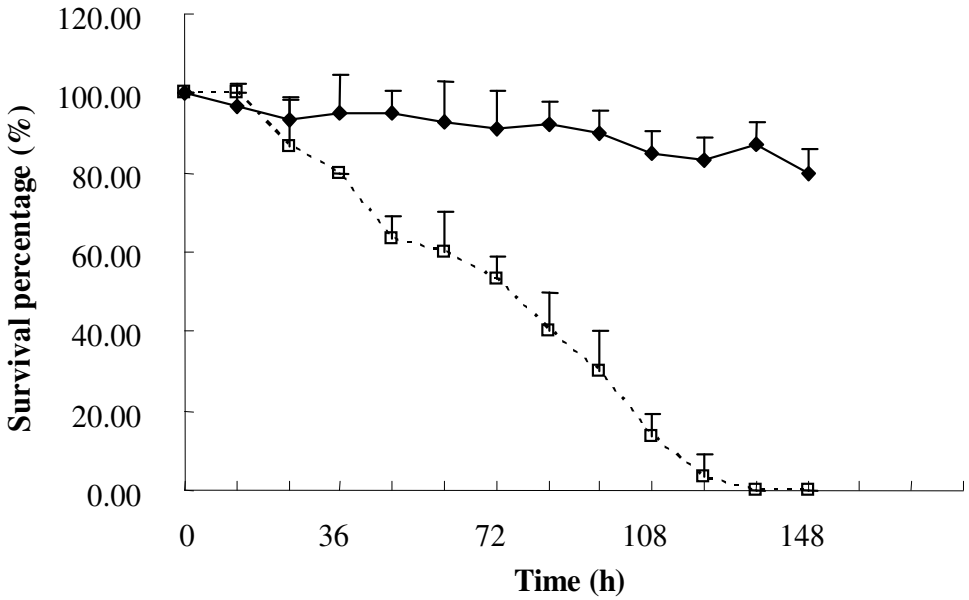

Figure 2. Survival ratio of WSSV-infected juvenile *F. chinensis* after being fed with WSSV positive *Artemia franciscana*. Fifty samples used in each group were carried out in 5 replicates. —□— WSSV-infected *F. chinensis*; —◆— control of *F. chinensis*.

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