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ORIGINAL ARTICLE

# Assessment of the role of brine shrimp *Artemia* in white spot syndrome virus (WSSV) transmission

**Jia-Song Zhang · Shuang-Lin Dong · Yun-Wei Dong · Xiang-Li Tian · Yi-Cheng Cao · Zuo-Jia Li · Dong-Chun Yan**

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**Abstract** Challenge tests with *Artemia* four different development stages (nauplii, metanauplii, pseudoadults and adults) to white spot syndrome virus was carried out by immersion challenge and virus-phytoplankton adhesion route in order to asses the possibility of *Artemia* acting as a vector of WSSV to penaeid shrimp *Litopenaeus vannamei* postlarvae. The WSSV succeeded in infecting four stages *Artemia*, and nested-PCR detection for WSSV revealed positive results to virus-phytoplankton adhesion route. No mass mortalities were observed in penaeid shrimp postlarvae fed with WSSV-positive *Artemia* which exposed to WSSV by virus-phytoplankton adhesion route, whereas WSSV DNA detected in penaeid shrimp postlarvae by nested-PCR. By contrary, no WSSV-positive was detected in any animal fed with WSSV-negative *Artemia*. These results indicated that *Artemia* could serve as a vector in WSSV transmission.

**Keywords** *Artemia* · WSSV · Vector · Transmission · *Litopenaeus vannamei*

## Introduction

Virus is an abundant microorganism in marine environment and hence diseases caused by virus are common in marine organisms. These diseases create great problems for commercial farming of marine organisms. One of the widest spread and most disastrous diseases for penaeid shrimp is white spot syndrome virus (WSSV) which first appeared in

J. S. Zhang · S. L. Dong (✉) · Y. W. Dong · X. L. Tian
The Key Laboratory of Mariculture, Ministry of Education Fisheries College,
Ocean University of China, Qingdao 266003, People's Republic of China
e-mail: dongsl@mail.ouc.edu.cn

J. S. Zhang · Y. C. Cao · Z. J. Li
South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences,
Guangzhou 510300, People's Republic of China

D. C. Yan
Department of Life Science, Ludong University, Yantai 264025, People's Republic of China

1993 (Huang et al. 1995a, b; Chou et al., 1995; Lightner 1996). Because of a most devastating and virulent viral agent, WSSV causes globe serious economic losses in penaeid shrimp culture industry, as 100 % cumulative mortalities can be reached within 3-10 days under farming conditions (Chou et al., 1995; Lightner 1996). The host range of the virus among crustaceans is remarkably wide, including many species of shrimp, crayfish, crab and lobster not only in seawater but also in freshwater (Huang et al., 1995b; Lo et al., 1996a, b, 1997; Peng et al., 1998a, b; Chou et al., 1995; Wang et al., 1998; Liu et al., 2000; Sahul Hameed et al., 2001, 2002). Based on recent researches, the virus was now assigned to the genus *Whispovirus*, family *Nimaviridae* (van Hulten et al., 2001; Mayo, 2002; Vlak et al., 2004).

Brine shrimp *Artemia* as live feed is widespread in marine shrimp hatcheries because of its nutritional and operational advantages (Léger et al., 1986). In recent years, however, more and more researches found that *Artemia* could be vector or host of some pathogens (Nicolas et al., 1989; Mortensen et al., 1993; Skliris et al., 1998; Bergh et al., 2001). Since WSSV-positive *Artemia* was detected in epizootiological surveys, *Artemia* is also under suspicious of acting as a reservoir or carrier of WSSV (Huang et al., 1995b; Song et al., 2001; Chang et al., 2002; Li et al., 2004; Deng et al., 2005). However, there is contradiction between different researches. Sahul Hameed et al. (2002) used immersion and oral route to test the pathogenicity of WSSV to *Artemia* five different development stages (nauplii, metanauplii, juveniles, sub-adults and adults) and one step PCR results showed the WSSV failed to infect them. Li et al. (2004) found that *Artemia* and their reproductive cysts become WSSV positive by one step PCR. However, the virus was undetectable by PCR in nauplii that had hatched from PCR-positive reproductive cysts. So they deduced that WSSV can be vertically transmitted from WSSV positive instars to reproductive cysts, but this WSSV is removed during hatching.

The aim of the present study was to investigate the pathogenicity of WSSV on four different developmental stages of *Artemia* and the role of *Artemia* in WSSV transmission.

## Materials and methods

### Test animals

Brine shrimp *Artemia* cysts were obtained from Changlu Huanghua Saltern of Hebei Province, China. The cysts were hatched in sterilized seawater (salinity 32 ppt) at 25°C. After 24-h incubation, hatched nauplii were separated from the unhatched and empty cysts, and stocked in 10-L aquariums with sterilized seawater and continuous aeration. The nauplii were fed on *Isochrysis zhanjiangensis* and reared to adult stage. Required stages nauplii, metanauplii, pseudoadults and adults (Liao et al. 1990) of *Artemia* were taken from this stock for experimental purposes.

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).

### Preparation of viral inoculum

WSSV-infected *Fenneropenaeus chinensis* with prominent white spots on the exoskeleton were collected from farms located at Rushan, China. Soft tissue (15 g) from the cephalothorax, including gills, was homogenized in 150 ml PBS and centrifuged at 3000 × *g* for 10 min at 4°C. The supernatant fluid was re-centrifuged at 8000 × *g* for 10 min at 4°C. The final supernatant

fluid was then filtered through a 0.4-μm filter before storage at -80°C until used. Before storage, the presence of WSSV in the tissue sample and the final supernatant fluid was determined by nested-PCR assay.

## Infection experiment

The WSSV experimental infection design for WSSV challenge followed that of Sahul Hameed et al. (2002) except of the challenge test with *Artemia* with virus-phytoplankton adhesion route and immersion challenge.

### *WSSV experimental infection with Artemia*

Challenge tests with *Artemia* at stage nauplii, metanauplii, pseudoadults and adults were sequentially carried out by immersion challenge and virus-phytoplankton adhesion route. Each treatment was conducted in triplicate. Healthy nauplii, metanauplii, pseudoadults and adults of *Artemia*, at 2000, 500, 200 and 100 ones $L^{-1}$, respectively, were reared separately in sterilized 1-L beakers with 800ml aerated seawater. The beakers were covered to prevent cross-contamination.

In the immersion challenge, the WSSV filtrate was introduced to the water at a volume equal to 0.1% of the total rearing medium (0.8ml/800ml) (Sahul Hameed et al., 2002). After a 3-h immersion, *Artemia* were washed three times and placed in fresh sterile seawater and *I. zhanjiangensis* provided. This immersion challenge was carried out at 07:00 and 19:00 h every day for 5 d. On Day 6 and Day 7, *I. zhanjiangensis* was provided twice daily, and the seawater was changed 100% daily. Before sample the animals starved for 24-h in order to empty alimentary canal.

For the virus-phytoplankton adhesion route, the method was carried out according to Zhang et al. (2006, 2007, 2008). In briefly, *I. zhanjiangensis* firstly was mixed with the viral supernatant for 0.5 h and then fed on *Artemia* twice a day for 5 d. On Day 6, and each day thereafter, the replicates received the same treatment as the immersion challenge groups.

The control groups received the same treatment as the immersion challenge, except they were exposed to PBS, not WSSV filtrate.

### *WSSV experimental infection with penaeid shrimp L. vannamei postlarvae*

Penaeid shrimp *L. vannamei* postlarvae (15 per beaker, body length 1-1.5 cm) were divided into four groups and maintained separately in 1-L beakers with 800ml sterilized seawater. All beakers were kept in an illuminated incubator at 25°C with a 12-h photoperiod. Each treatment was conducted in triplicates. In group I, the postlarvae were fed with *Artemia* unexposed to WSSV; in group II, the animals were fed with *Artemia* exposed to WSSV by immersion challenge; in group III, the animals were fed with *Artemia* exposed to WSSV by virus-phytoplankton adhesion route; in group IV, the animals were fed with minced WSSV-infected *F. chinensis* tissue. The animals were fed respective feeds at 5% of shrimp body weight twice a day for 15-d. Before sample the animals starved for 24-h in order to empty alimentary canal.

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 10.0 (SPSS Inc.).

**Table 1** Primers used for each WSSV-PCR detection method

| Primer name | Primer sequences (5'→3') | Product size (bp) |
|---|---|---|
| PB | AGCTAGGTATAGTGGCTGTTG | 1221 |
| PE | CTTCTGTGTGTGTCTACATCC | |
| P1 | ATCATGGCTGCTTCACAGAC | 982 |
| P2 | GGCTGGAGAGGACAAGACAT | |

## Molecular of diagnosis WSSV infection

WSSV infection in *Artemia* and *L. vannamei* postlarvae was confirmed by nested-PCR using primers designed by Kong et al. (2003). The first set of primers (PB and PE), external, amplifies a 1221-bp fragment and the second set of primers (P1 and P2), internal, amplifies a 982-bp fragment of the WSSV genome (Table 1). The PCR amplification for 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 $MgCl_2$, 15 mM each primer, 200 mM each 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 initially denaturation 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, visualized under UV light and then photographed.

To test nauplii, metanauplii, pseudoadults and adults of *Artemia*, pools of 50, 30, 20, 10 individuals, respectively, were fixed together and used for PCR determination. For *L. vannamei* postlarvae, two survive or dead ones' muscles (0.05g) fixed together in one 1.5ml centrifuge tube for determination.

## DNA extraction

DNA extraction of *Artemia* and *L. vannamei* postlarvae was carried out according to the method of Wang et al. (2000). Materials were fixed in SEMP–Tris (10 mM Tris–HCl,

**Fig. 1** Nested-PCR detection of WSSV DNA in *Artemia* exposed to WSSV by immersion challenge or virus-phytoplankton adhesion route. Lane M: marker; Lane P: positive control; Lane N: negative control; Lane 1: *Artemia* nauplii in control group; Lane 2: *Artemia* metanauplii in control group; Lane 3: *Artemia* pseudoadults in control group; Lane 4: *Artemia* adults in control group; Lane 5: *Artemia* nauplii in immersion challenge group; Lane 6: *Artemia* metanauplii in immersion challenge group; Lane 7: *Artemia* pseudoadults in immersion challenge group; Lane 8: *Artemia* adults in immersion challenge group; Lane 9: *Artemia* nauplii in virus-phytoplankton adhesion route group; Lane 10: *Artemia* metanauplii in virus-phytoplankton adhesion route group; Lane 11: *Artemia* pseudoadults in virus-phytoplankton adhesion route group; Lane 12: *Artemia* adult in virus-phytoplankton adhesion route group

**Fig. 2** Nested-PCR detection of WSSV-DNA in *Litopenaeus vannamei* postlarvae fed with different feeds. Lane M: marker; Lane N: negative control; Lane P: positive control ; Lanes A–C: *L. vannamei* postlarvae fed with brine shrimp *Artemia* unexposed to WSSV; Lanes D–F: *L. vannamei* postlarvae fed with *Artemia* in immersion challenge group; Lanes G–I: *L. vannamei* postlarvae fed with *Artemia* in virus-phytoplankton adhesion route group; Lanes J–L: *L. vannamei* postlarvae fed with minced WSSV-infected *F. chinensis* tissue

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.

## Results and discussion

WSSV-positive was not revealed in control and immersion groups, but it was observed in virus-phytoplankton adhesion route group following nested-PCR amplification with the internal set primers (Fig. 1). No WSSV-positive result showed by PCR amplification using the first set primers.

Among experimental groups, penaeid shrimp *L. vannamei* postlarvae fed with *Artemia* exposed to WSSV by virus-phytoplankton adhesion route and by fed with minced WSSV-infected penaeid shrimp tissue were found WSSV-positive by nested-PCR, whereas animals in other groups showed WSSV-negative (Fig. 2).

The mortalities in all groups increased steadily over a period of 15 days and, however, WSSV infection did not cause mass mortality in groups fed with *Artemia* except in groups fed with WSSV infected penaeid shrimp tissue (Table 2.). There was no significant difference among groups of penaeid shrimps fed with *Artemia* ( $P > 0.05$).

In epizootiological surveys and experimental infection, WSSV-positive was detected on *Artemia* cysts and adults (Huang et al.,1995b; Song et al., 2001; Sahul Hameed et al., 2002; Chang et al. 2002; Li et al. 2004; Deng et al., 2005). The present experiment gave similar

**Table 2** The experiment of penaeid shrimp *Litopenaeus vannamei* postlarvae fed with brine shrimp *Artemia* exposed to WSSV by different challenge way

| Group | Feeding regimen | Cumulative mortality(%) | Detection by nested-PCR |
|---|---|---|---|
| I | Fed with *Artemia* not exposed to WSSV | 17.78 ± 3.85 | - |
| II | Fed with *Artemia* exposed to WSSV by immersion challenge | 13.33 ± 6.67 | - |
| III | Fed with *Artemia* exposed to WSSV by virus-phytoplankton adhesion route | 13.33 ± 0.00 | + |
| IV | Fed with minced WSSV-infected *F. chinensis* tissue | 100.00 ± 0.00 | + |

-: WSSV-negative; +: WSSV-positive

results, which indicated that *Artemia* could be infected by WSSV and became carrier of the virus.

Sahul Hameed et al. (2002) used immersion and oral route to test the pathogenicity of WSSV to *Artemia* five different development stages (nauplii, metanauplii, juveniles, sub-adults and adults) and one step PCR results showed the WSSV failed to infect them. In the oral route of infection, *Artemia* were fed with the mixture of rice bran and WSSV. However, Li et al. (2004) fed *Artemia* with viral feeding mixture (VFM) in which WSSV were mixed with powdered algae. Then, Li et al. (2004) found that *Artemia* and their reproductive cysts become WSSV positive by one step PCR. Therefore, different infection ways bring different results. In the present experiment, using nested-PCR, WSSV-positive was revealed in *Artemia* nauplii, metanauplii, pseudoadults and adults exposed to WSSV by virus-phytoplankton adhesion route (Fig. 1). These results indicated that virus-phytoplankton adhesion route is an efficient transmission route of WSSV to zooplankton, and similar results also were reported in rotifer *Brachionus urceus* and harpacticoid copepod *Nitocra* sp. (Zhang et al. 2006, 2007, 2008). Using this method, WSSV initially adhered to microalga, and *Artemia*, filter feeder, may ingest and accumulate virus in their alimentary canal. Therefore, it is also possible that WSSV are carried passively in the digestive tracts of *Artemia* and transmitted to penaeid shrimp by oral route (Fig. 2, Table 2).

The mortality of penaeid shrimp *L. vannamei* postlarvae, fed with WSSV-positive *Artemia*, increased steadily during the experiment, WSSV infection did not cause mass mortality (Table 2), which may due to low quantity of virus inside shrimp. However, the present experiment results still imply there is a possibility that these WSSV-positive penaeid shrimp postlarvae lead to virus prevalence in later time, which might explain mass mortalities in penaeid shrimp ponds after traditional stocking postlarvae. Those postlarvae, infected WSSV by fed with infected or contaminated food in hatcheries, become potentially source of viral loads. When these infected larvae are stocked to rearing pond, they would meet various WSSV-positive aquatic organisms, especially zooplankton, and prey on them. So more WSSV would be accumulated inside shrimp larvae and WSS could be triggered under various kind stresses. When WSS initially appears, the moribund larvae can be cannibalized and a successive mortality peak will appear. Therefore, mass mortalities are often observed by farmers after shrimp larvae stocked in rearing pond 30–40 days latter.

Even *Artemia* has restrictive infection ability in WSSV transmission, its ability in the virus transmission can not be overlooked because *Artemia* is being widely used as an important living feed in crustacean hatcheries. How long would WSSV keep infective ability inside *Artemia* should be valued in further experiment.

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