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Studies on the rotifer (*Brachionus urceus* Linnaeus, 1758) as a vector in white spot syndrome virus (WSSV) transmission

Jia-Song Zhang [a], Shuang-Lin Dong [a,*], Xiang-Li Tian [a], Yun-Wei Dong [a], Xiang-Yi Liu [a,b], Dong-Chun Yan [c]

[a] *The Key Laboratory of Mariculture, Ministry of Education Fisheries College, Ocean University of China, Qingdao 266003, PR China*
[b] *Science and Technology Bureau of Rizhao, Rizhao 276826, PR China*
[c] *Department of Life Science, Ludong University, Yantai 264025, PR China*

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Abstract

The rotifer *Brachionus urceus* (Linnaeus, 1758) was experimentally infected with the white spot syndrome virus (WSSV) by the virus–phytoplankton adhesion route in order to assess the possibility of rotifer acting as a vector of WSSV to infect the shrimp *Fenneropenaeus chinensis* (Osbeck, 1765) larvae at zoea stage III. The nested-PCR test revealed WSSV-positive results in the rotifers exposed to WSSV by the virus–phytoplankton adhesion route. Among 10 replicates in the infection treatment, 40% of *F. chinensis* larvae became WSSV-positive when fed with WSSV-positive rotifers, whereas all were WSSV-negative for *F. chinensis* when fed with WSSV-free rotifers. Though the mortality of shrimp larvae in the infection treatment (39.47±15.44%) was higher than that in the control treatment (34.67±15.11%), there was no significant difference in the mortality between them ($P>0.05$). These results indicated that the rotifer could serve as a vector in WSSV transmission when ingested.

Keywords: White spot syndrome virus; Rotifer; Vector; Transmission; *Fenneropenaeus chinensis*

1. Introduction

White Spot Syndrome Virus (WSSV) is 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., 2004). The first outbreak of WSSV was reported in Japan in 1993 (Inouye et al., 1994; Momoyama et al., 1994, 1995; Nakano et al., 1994) and WSSV is now one of the most devastating and virulent viral agents threatening the penaeid shrimp culture industry. Shrimp cumulative mortality can be reached 100% within 3 to 10 days under farming conditions (Chou et al., 1995; Lightner, 1996).

The WSSV has a wide host range which includes copepods, shrimp, crayfish, crab, lobster, freshwater crab and prawn (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). In epizootiological surveys, many zooplanktonic species, including rotifers, present in penaeid shrimp rearing ponds were also found to be WSSV-positive using different diagnostic methods. These zooplanktonic species were suspected to be WSSV hosts or carriers

* Corresponding author. Tel.: +86 532 82032827; fax: +86 532 82032799.
E-mail address: dongsl@mail.ouc.edu.cn (S.L. Dong).

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(Huang et al., 1995; Lo et al., 1996a; Wang et al., 1998; He et al., 1999; Liu et al., 2000; Yan et al., 2004). Rotifers are an important zooplanktonic group as they are a key link in the energy flow between primary and secondary producers in shrimp ponds. They are also widely used as live feed in shrimp hatcheries worldwide due to their nutritional and operational advantages. In hatcheries, shrimp larvae are fed with rotifers from zoea stage to postlarval stage, where individual larvae consume approximately 50–100 rotifers per day. Previous works revealed that the rotifers were found to be responsible for the source of viral and bacterial infections in fish hatcheries (Eckmann, 1985; Tanasomwang and Muroga, 1988; Skliris and Richards, 1998).

The present study is aimed to assess the possibility that the rotifer may act as a reservoir or carrier of white spot syndrome virus to infect marine shrimp.

2. Materials and methods

2.1. Test animals

The rotifer *Brachionus urceu* was originally hatched from sediment samples of a shrimp rearing pond collected in 2003 from Rushan, China. After hatching, the population was cultured in 40-L aquariums filled with sterilized seawater (salinity 32 ppt) in an illuminated incubator (23 °C, 12-h photoperiod). The zooplankton fed on *Isochrysis zhanjiangensis* twice a day at a density of 1.2×10^6 cells ml^{-1}.

The hatchery of the Aquaculture Institute of Rizhao (Shangdong Province, PR China) provided the fertilized eggs of *Fenneropenaeus chinensis*. The fertilized eggs were then hatched in the Key Laboratory of Mariculture (Ocean University of China, Qingdao, PR China). Once larvae developed to zoea stage I, the animals were fed on *I. zhanjiangensis* four times daily at a density of 1.2×10^6 cells ml^{-1}.

The rotifers or shrimp larvae were screened by nested-PCR and WSSV-negative animals were used in the experiment.

2.2. Preparation of the viral inoculum

For the inoculum preparation, soft tissue (15 g) from the cephalothorax of WSSV-infected *F. chinensis* (with prominent white spots on the exoskeleton) was homogenized in 150 ml PBS (288.75 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PHO_4, pH 7.3), then centrifuged at 3000 ×*g* for 10 min at 4 °C. The supernatant was centrifuged at 8000 ×*g* for 10 min at 4 °C. The final supernatant was filtered through a 0.45-μm filter and stored 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 (Kong et al., 2003).

2.3. Infectivity experiment

2.3.1. Challenge of WSSV with the rotifer

The infectivity experiment of WSSV in rotifers comprised of two treatments: test and negative control. Each treatment consisted of 5 replicates. In each replicate, rotifers (5 ind. ml^{-1}) were stocked in sterile 1-L beakers containing 800 ml of sterilized seawater. Beakers of each treatment were kept separately in two illuminated incubators (23 °C, 12-h photoperiod) in order to prevent cross-contamination.

In the test treatment, rotifers were exposed to WSSV using the virus–phytoplankton adhesion route. *I. zhanjiangensis* (250 ml, 2.4×10^6 cells ml^{-1}) was first mixed with 2.5 ml of the viral inoculum for 0.5-h, and then divided into 5 aliquots to feed on the rotifers in five beakers. After 3-h, the rotifers were washed three times and placed in the fresh sterile seawater. The rotifers were fed at 07:00 and 19:00 every day for 3 days. At the end of the 4-d experiment period, animals left to starve for 24-h and sampled to determine WSSV infection. After sampling, *B. urceus* was fed with *I. zhanjiangensis* (1.2×10^6 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 *I. zhanjiangensis* first mixed with PBS, not the WSSV inoculum.

2.3.2. Infection of *F. chinensis* larvae with WSSV-positive rotifers

Infection of *F. chinensis* larvae with WSSV-positive rotifers also included an infection and control treatment, each being carried out in 10 replicates. *F. chinensis* larvae at zoea stage III (15–20 per beaker, body length about 2.5 mm) were acclimated individually in 250 ml beaker with 200 ml sterile seawater. Replicates of each treatment were kept separately in two illuminated incubators (23 °C, 12-h photoperiod) in order to prevent the cross-contamination. In the infection treatment, the larvae were fed with WSSV-positive rotifers twice a day at a density of 20 ind. ml^{-1}. In the control treatment, the larvae were fed with WSSV-negative rotifers at the same density and frequency as the infection treatment. Rotifers were filtered through a 75-μm screen, and then rinsed three times on the screen with sterile seawater before being fed to the shrimp larvae. The experiment lasted 9 days and the larvae left to starve for 24-h before sampling.

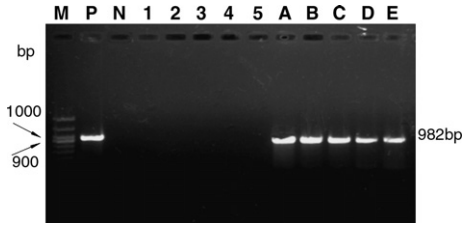

Fig. 1. Nested-PCR detection of WSSV in the rotifer *B. urceus* exposed to WSSV by virus–phytoplankton adhesion route. Lane M: marker; Lane P: positive control; Lane N: negative control; Lanes 1–5: *B. urceus* in the control treatment; Lanes A–E: *B. urceus* in the infection treatment in which the rotifers were exposed to WSSV by virus–phytoplankton adhesion route.

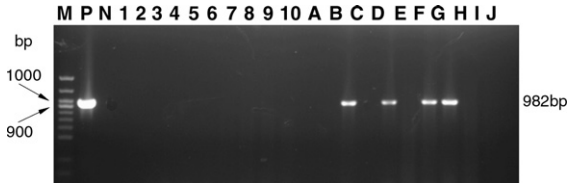

Fig. 2. Nested-PCR detection of WSSV in penaeid shrimp *F. chinensis* larvae at zoea stage III fed with WSSV negative or positive rotifers *B. urceus*. Lane M: marker; Lane P: positive control; Lane N: negative control; Lanes 1–10: *F. chinensis* larvae fed with WSSV-negative rotifers; Lanes A–J: *F. chinensis* larvae fed with WSSV-positive rotifers.

The larvae were examined twice a day. Dead larvae were removed and cumulative mortality was calculated. Mortalities were analyzed using the Analysis of Variance (ANOVA) following software SPSS 10.0.

2.4. DNA extraction

Rotifers of each treatment were pooled into 100–200 ind. for DNA extraction. In the infection experiment with *F. chinensis* larvae, survival and dead penaeid shrimp larvae were all fixed together for PCR determination.

Extraction of DNA from the rotifers and penaeid shrimp larvae was carried out according to the method of Wang et al. (2000) with slight modifications. Materials were fixed in SEMP-Tris (10 mM Tris–HCl, 70 mM EDTA, 1% SDS, 0.5% mercaptoethanol, phenol saturated, pH 8.0) and extracted by boiling followed by an ethanol precipitation. Dried DNA was dissolved with Tris-ethylenediaminetetraacetic acid (TE) (pH 8.0) buffer.

Table 1
Nested-PCR analysis results of WSSV infection with the rotifer *B. urceus* and penaeid shrimp *F. chinensis* larvae at zoea stage III

Test animals	Nested-PCR positive	Prevalence (%)
B. urceus in the control treatment	0/5	0
B. urceus in the infection treatment in which the rotifers were exposed to WSSV by virus–phytoplankton adhesion route	5/5	100
F. chinensis larvae fed with WSSV-negative rotifers *B. urceus*	0/10	0
F. chinensis larvae fed with WSSV-positive rotifers exposed to WSSV by virus–phytoplankton adhesion route	4/10	40

2.5. Molecular diagnosis of WSSV infection

WSSV infection in rotifers and penaeid shrimp larvae was confirmed by nested-PCR (Kong et al., 2003). The first set of primers (internal primers) amplified a 1221-bp fragment while the second set of primers (internal primers) amplified a 982-bp fragment of the WSSV genome. The PCR amplification was carried out in a 25 μl reaction mixture containing 1 μl template DNA (approximately 150 ng), 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 deionized water to make up the final volume. PCR cycle conditions set at 94 °C for 5 min for the initial denaturation step, followed by 30 cycles with 40 s at 94 °C for denaturation, 40 s at 58 °C for annealing, 2 min at 72 °C for extension and a final extension at 72 °C for 10 min. After amplification, an aliquot of the PCR product was analyzed by electrophoresis in 0.8% agarose gel stained with ethidium bromide (0.5 μg ml^{-1}) and visualized by ultraviolet transillumination.

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.

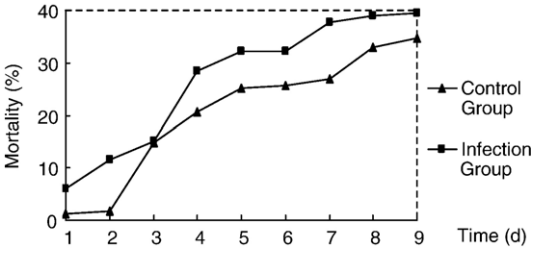

Fig. 3. Mean cumulative mortality per day of penaeid shrimp *F. chinensis* larvae at zoea stage III exposed to WSSV by fed with WSSV negative or positive rotifers *B. urceus*.

3. Results and discussion

Rotifers of the infection treatment showed WSSV-positive using the nested-PCR method when the animals were exposed to WSSV by the virus–phytoplankton adhesion route. No positive results were revealed in the control treatment (Fig. 1, Table 1).

Phytoplankton are the base of the food web in pond cultures. In addition, they remove small particles in culture water, including viruses which have been excreted by infected animals, to maintain a stable environment condition for culture (Boyd and Tucker, 1998). Therefore, we hypothesize that phytoplankton can carry WSSV (Liu, 2003). This implies that filter feeders, especially zooplankton, ingest phytoplankton which carried WSSV, and therefore accumulate viral particles. The present experiment results revealed that the filter feeding habit of the animals might be responsible for making WSSV-positive in the case of rotifer (Fig. 1, Table 1).

In the infection treatment, 40% of *F. chinensis* larvae became WSSV-positive through feeding WSSV-positive rotifers and others were WSSV-negative, whereas larvae fed with WSSV-free rotifers were all WSSV-negative by nested-PCR analysis (Fig. 2, Table 1). Our results indicate that rotifers may serve as a vector in WSSV transmission to penaeid shrimp larvae when ingested (Fig. 2, Table 1).

Though the mortality of the shrimp larvae in the infection treatment (39.47±15.44%) was higher than that of the control treatment (34.67±15.11%), there was no significant difference between them ($P>0.05$). In addition, the mortalities in the two treatments increased steadily over a period of 9-d. However, mass mortality was not present in the infection treatment (Fig. 3). This could be due to the low susceptibility of the shrimp larvae to WSSV before postlarval stage (PL). Other studies have also shown that mass mortality caused by WSSV could not be detected before PL 10 (Lightner et al., 1998; Venegas et al., 1998; Pramod Kiran et al., 2002; Yoganandhanl et al., 2003; Perez et al., 2005).

In conclusion, it appears that transmission via infected rotifer ingestion is a viable vector for WSSV. Further work should be done to identify the roles of other zooplanktonic species, especially copepods, in WSSV transmission.

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References

Boyd, C.E., Tucker, C.S., 1998. Pond Aquaculture Water Quality Management. Kluwer, Boston.

Chen, L., Lo, C.F., Chiu, Y.L., Chen, F.C., Kou, G.H., 2000. Experimental infection of white spot syndrome virus WSSV in benthic larvae of mud crab *Scylla serrata*. Dis. Aquat. Org. 40, 157–161.

Chou, H.Y., Huang, C.Y., Wang, C.H., Chiang, H.C., Lo, C.F., 1995. Pathogenicity of a baculovirus infection causing white spot syndrome in culture penaeid shrimp in Taiwan. Dis. Aquat. Org. 23, 165–173.

Chou, H.Y., Huang, C.Y., Lo, C.F., Kou, G.H., 1998. Studies on transmission of white spot syndrome associated baculovirus (WSBV) in *Penaeus monodon* and *P. japonicus* via waterborne contact and oral ingestion. Aquaculture 164, 263–276.

Eckmann, R., 1985. Histopathological alterations in the intestine of whitefish Coregonus sp. larvae reared on zooplankton from Lake Constance. Dis. Aquat. Org. 1, 11–17.

He, J.G., Zhou, H.M., Yao, B., Yang, X.M., Deng, M., 1999. White spot syndrome baculovirus (WSBV) host range and transmission route. Acta Sci. Nat. Univ. Sunyatseni 38, 65–69 (in Chinese with English abstract).

Huang, J., Yu, J., Wang, X.H., Song, X.L., Ma, C.S., Zhao, F.Z., Yang, C.H., 1995. Survey on the pathogen and route of transmission of baculoviral hypodermal and hematopoietic necrosis in shrimp by ELISA of monoclone antibody. Mar. Fish. Res. 16, 40–50 (in Chinese with English abstract).

Inouye, K., Miwa, S., Oseko, N., Nakano, H., Kimura, T., 1994. Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: electron microscopic evidence of the causative virus. Fish. Pathol. 29, 149–158.

Kong, J., Liu, P., Shi, T., Xiang, J.H., 2003. A baculovirus DNA fragment sequencing from *Penaeus chinensis*. Acta Oceanol. Sin. 25, 186–189 (in Chinese with English abstract).

Lightner, D.V., 1996. A Handbook of Pathology and Diagnostic Procedures for Diseases of Penaeid Shrimp. World Aquaculture Society, Baton Rouge, LA.

Lightner, D., Hason, K., White, B., Redman, R., 1998. Experimental infection of western hemisphere penaeid shrimps with Asian white spot syndrome virus and Asian yellow head virus. J. Aquat. Anim. Health 10, 271–281.

Liu, B. 2003. The roles of salinity and microalgae on infectivity of white spot syndrome virus (WSSV) in penaeid shrimp. PhD Thesis (in Chinese with English abstract).

Liu, P., Kong, J., Meng, X.H., Liu, Z.H., Li, J., 2000. Investigation of the transmission route during the artificial culture of shrimp with the white spot syndrome virus. Mar. Fish. Res. 21, 9–12 (in Chinese with English abstract).

Lo, C.F., Ho, C.H., Peng, S.E., Chen, C.H., Hsu, H.C., Chiu, Y.L., Chang, C.F., Liu, K.F., Su, M.S., Wang, C.H., Kou, G.H., 1996a. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis. Aquat. Org. 27, 215–225.

Lo, C.F., Leu, J.H., Ho, C.H., Chen, C.H., Peng, S.E., Chen, Y.T., Chou, C.M., Yeh, P.Y., Huang, C.J., Chou, H.Y., Wang, C.H., Kou, G.H., 1996b. Detection of baculovirus associated with white spot

syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis. Aquat. Org. 25, 133–141.

Lo, C.F., Ho, C.H., Chen, C.H., Liu, K.F., Chiu, Y.L., Yeh, P.Y., Peng, S.E., Hsu, H.C., Liu, H.C., Chang, C.F., Su, M.S., Wang, C.H., Kou, G.H., 1997. Detection and tissue tropism of white-spot syndrome baculovirus (WSBV) in captures brooders of *Penaeus monodon* with a special emphasis on reproductive organs. Dis. Aquat. Org. 30, 53–72.

Lo, C.F., Chang, Y.S., Cheng, C.T., Kou, G.H., 1998. PCR monitoring of cultured shrimp for white spot syndrome virus (WSSV) infection in growout ponds. In: Flegel, T.W. (Ed.), Advances in Shrimp Biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok, pp. 281–286.

Maeda, M., Itami, T., Furumoto, A., Hennig, O., Imamura, T., Kondo, M., Hirono, I., Aoki, T., Takahashi, Y., 1998. Detection of penaeid rod-shaped DNA virus (PRDV) in wild-caught shrimp and other crustaceans. Fish Pathol. 33, 373–380.

Mayo, M.A., 2002. A summary of taxonomic changes recently approved by ICTV. Arch. Virol. 147, 1655–1656.

Momoyama, K., Hiraoka, M., Nakano, H., Koube, H., Inouye, K., Oseko, N., 1994. Mass mortalities of cultured Kuruma shrimp, *Penaeus japonicus* in Japan in 1993: histopathological study. Fish Pathol. 29, 141–148.

Momoyama, K., Hiraoka, M., Inouye, K., Kimura, T., Nakano, H., 1995. Diagnostic techniques of the rod-shaped nuclear virus infection in the Kuruma shrimp, *Penaeus japonicus*. Fish Pathol. 30, 263–269.

Nakano, K, Kuobe, H., Umezawa, S., Momoyama, K., Hiraoka, M., Inouye, K., Oseko, S., 1994. Mass mortalities of cultured Kuruma shrimp *Penaeus japonicus* in Japan in 1993: epizootiological survey and infection trials. Fish Pathol. 29, 135–139.

Peng, S.E., Lo, C.F., Ho, C.H., Chang, C.F., Kou, G.H., 1998. Detection of white spot baculovirus (WSBV) in giant freshwater prawn, *Macrobrachium rosenbergii*, using polymerase chain reaction. Aquaculture 164, 253–262.

Perez, F., Volckaert, F.A.M., Calderon, J., 2005. Pathogenicity of white spot syndrome virus on postlarvae and juveniles of *Penaeus* (*Litopenaeus*) *vannamei*. Aquaculture 205, 586–591.

Pramod Kiran, R.B., Rajendran, K.V., Jung, S.J., Oh, M.J., 2002. Experimental susceptibility of different life-stages of the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man), to white spot syndrome virus (WSSV). J. Fish Dis. 25, 201–208.

Sahul Hameed, A.S., Yoganandhanl, K., Sathish, S., Rasheed, M., Murugan, V., Jayaraman, K., 2001. White spot syndrome virus WSSV in two species of freshwater crabs *Paratelphusa hydrodomous* and *P. pulvinata*. Aquaculture 201, 179–186.

Sahul Hameed, A.S., Murthi, B.L.M., Rasheed, M., Sathish, S., Yoganandhanl, K., Murugan, V., Jayaraman, K., 2002. An investigation of Artemia as a possible vector for white spot syndrome virus (WSSV) transmission to *Penaeus indicus*. Aquaculture 204, 1–10.

Sahul Hameed, A.S., Balasubramanian, G., Musthaq, S.S., Yoganandhanl, K., 2003. Experimental infection of twenty species of Indian marine crabs with white spot syndrome virus (WSSV). Dis. Aquat. Org. 57, 157–161.

Skliris, G.P., Richards, Randolph H., 1998. Assessment of the susceptibility of the brine shrimp *Artemia salina* and rotifer *Brachionus plicatilis* to experimental nodavirus infections. Aquaculture 169, 133–141.

Tanasomwang, V., Muroga, K., 1988. Intestinal microflora of larval and juvenile stages in Japanese flounder (*Paralichthys olivaceus*). Fish Pathol. 23 (2), 77–83.

Van Hulten, M.C., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., Sandbrink, H., Lankhorst, R.K., Vlak, J.M., 2001. The white spot syndrome virus DNA genome sequence. Virology 286, 7–22.

Venegas, C., Nonaka, L., Nushiake, K., Shimizu, K., Nishizawa, T., Muroga, K., 1998. Pathogenicity of penaeid, rod-shaped DNA virus (PRDV) to Kuruma prawn in different developmental stages. Fish Pathol. 34, 19–23.

Vlak, J.M., Bonami, J.R., Flegel, T.W., Kou, G.H., Lightner, D.V., Lo, C.F., Loh, P.C., Walker, P.W., 2004. Nimaviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), VIIIth Report of the International Committee on Taxonomy of Viruses. Elsevier, Amsterdam, pp. 187–192.

Wang, Y.C., Lo, C.F., Chang, P.S., Kou, G.H., 1998. Experimental infection of white spot baculovirus in some cultured and wild decapods in Taiwan. Aquaculture 164, 221–231.

Wang, K., Shi, C.Y., Huang, J., 2000. The comparison of different methods of DNA extracting applied in PCR detection of hypodermal and hematopoietic necrosis baculovirus. Mar. Fish. Res. 21, 8–12 (in Chinese with English abstract).

Yan, D.C., Dong, S.L., Hang, J., Yu, X.M., Feng, M.Y., Liu, X.Y., 2004. White spot syndrome (WSSV) detected by PCR in rotifer and rotifer resting eggs from shrimp pond sediments. Dis. Aquat. Org. 59, 69–73.

Yoganandhan1, K., Narayanan1, R.B., Sahul Hameed, A.S., 2003. Larvae and early post-larvae of *Penaeus monodon* (Fabricius) experimentally infected with white spot syndrome virus (WSSV) show no significant mortality. J. Fish Dis. 26, 385–391.