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Evidence of WSSV transmission from the rotifer *(Brachionus plicatilis)* to the black tiger shrimp (*Penaeus monodon*) postlarvae and means to control rotifer resting eggs using industrial disinfectants

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Abstract. Rotifers are considered possible vectors of the white spot syndrome virus (WSSV) and have been implicated in its recurrence in pond-cultured shrimp. However, direct evidence of the transmission and the pathogenicity of this virus from rotifers to shrimp has been lacking. In the present study, the pathogenicity of WSSV transmitted from infected rotifers (*Brachionus plicatilis*) to post larval black tiger shrimp (*Penaeus monodon)* was investigated. Results show that WSSV transmitted from infected rotifers to post-larval *P. monodon* caused an 82% cumulative mortality as compared to a 9% mortality in the non-infected control group. We also investigated the possibility of industrial disinfectants (sodium hypochlorite, granulated calcium hypochlorite and formalin) as possible means to inhibit the viability of rotifer resting eggs, considered a biological reservoir of WSSV in earthen ponds. Among the disinfectants that were tested, granulated calcium hypochlorite at 5 mg/L was the most effective. The present study provides direct evidence of the high pathogenicity of WSSV transmitted from rotifers to post larval *P. monodon* and shows the potential use of granulated calcium hypochlorite in pond disinfection. This treatment could be a promising strategy to inhibit the spread and recurrence of WSSV outbreaks in *P. monodon* culture.

Key Words: rotifers, resting eggs, WSSV, disinfectants.

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Introduction

The white spot syndrome virus (WSSV) of the genus *Whispovirus*, family Nimaviridae is the major cause of mass mortalities and economic losses in shrimp aquaculture. This virus is highly pathogenic that could kill all infected shrimp within 7 to 10 days post infection (Chou *et al* 1995; Lightner 1996). Other than shrimp, WSSV is also known to infect other crustacean species including crabs and crayfishes (Wang *et al* 1998; Lo *et al* 1996).

Recently, Yan *et al* (2004) reported that rotifers, commonly found in the rearing water of cultured shrimp are also host of WSSV and a possible vector in the transmission of this virus. Further, the work of Zhang *et al* (2006) provided direct evidence of the transmission of WSSV from infected *Brachionus urceus* to the larvae of *Penaeus chinensis*. Rotifers also posses the unique ability to produce resting eggs enclosed in a hard protective casing that serves as a protective barrier against adverse environmental conditions and chemical treatments. These resting eggs can stay viable for months and even years in sediments of shrimp culture ponds, serve as biological reservoir of the virus and could be a probable cause of WSSV recurrence.

Since the first report of WSSV occurrence in the Philippines in 2000 (Magbanua *et al* 2000), the virus has been implicated in yearly crop losses of pond cultured *P. monodon*.

Rotifers could be a possible vector and reservoir of WSSV in earthen ponds but information on the transmission and pathogenicity of WSSV from infected rotifers to *P. monodon* has been lacking. Also, there is a need to develop a disinfection method to kill and eliminate rotifers resting eggs to reduce the risk and recurrence of this disease in *P. monodon* culture. The present study detected WSSV in water rotifer and examined the transmission and pathogenicity of WSSV from infected rotifers to post larval *P. monodon*. In addition the efficacies of 3 different commercially available disinfectants (NaOCl, $Ca(OCl)_2$, H_2CO), to destroy the resting eggs of rotifers were also tested.

Materials and Methods

Artificial infection of rotifer (*Brachionus plicatilis*) with WSSV

WSSV was prepared from a stored, deep frozen (-80˚C freezer) tissue of a *P. monodon* infected with WSSV. One gram of infected shrimp gill was macerated with phosphate buffered saline (PBS, pH 8.2) and passed through a cellulose filter (0.45 µm, Millipore, USA). The resulting filtrate was centrifuged at 10,000 xg at 4˚C for 10 minutes (Eppendorf, Germany) before being injected intramuscularly into the 2nd or 3rd pleopod of apparently healthy shrimp (1.02 g, WSSV- negative). Antibiotic or antimycotic treatment was omitted as the filter can effectively exclude bacteria into the filtrate. Dead shrimp with evident white spots in the carapace of the cephalothorax area were collected, confirmed by PCR for WSSV infection and stored at -80˚C. Viral inoculum used in the rotifer infection experiment was prepared in a similar manner as described above in the preparation of WSSV inoculum for shrimp infection.

Newly-hatched rotifers were collected in a 1-L beaker. The rotifer stock was then divided into two containers with a density of 500 ind mL^{-1} each as the infected and non-infected stock. For the infected stock rotifers (positive control), 5 mL of the filtered WSSV supernatant was added to the first container and aerated for 2 hours. For the non-infected rotifers (negative control), the other container was added with 5 mL PBS. The two containers were covered with foil to prevent contamination. After 2 hours, rotifers were collected and washed three times with sterile seawater (SSW). Sterile seawater was prepared by autoclaving for 15 minutes at 15 psi. Samples for both infected and non-infected rotifers were collected for PCR detection.

Pathogenicity and transmission of WSSV from infected rotifers to *P. monodon* larvae

To assess the transmission and pathogenicity of WSSV from rotifers to shrimp, 200 *P. monodon* postlarvae (PL 21, 12.75 mm) were equally distributed in ten 5-L round glass aquarium at a density of 20 post larvae per aquarium. The experiment was conducted in two experimental treatments consisting of shrimp co-cultured with WSSV-negative rotifers and shrimp co-cultured with WSSV infected rotifers. Each treatment was replicated five times. Seawater used (28.8˚C, 32 ppt) was first passed through a sand filter then to a UV lamp for disinfection. Aeration was provided and each aquarium was covered with foil to prevent cross-contamination. Monitoring was done twice daily with dead and moribund larvae collected and stored in -80˚C. Mortalities were pooled and expressed as % cumulative mortality. After 9 days, the remaining shrimp were collected and processed for PCR analysis. The experiment was conducted at the Infection Building of SEAFDEC-Aquaculture Department, Tigbauan, Iloilo.

WSSV detection by PCR

Samples were collected and DNA was extracted following the procedures of Caipang *et al* (2011). Tissue sample was homogenized and centrifuged for 15 minutes at 12,000 rpm (4˚C). The supernatant was collected, added with 100% ethanol and concentrated by centrifugation for 10 minutes at 12,000 rpm (4˚C). The supernatant was decanted and the resulting DNA pellet was solubilized in 1x TE buffer and stored at -20°C for subsequent PCR analyses.

DNA samples were tested for the presence of WSSV using a PCR technique based on the protocol developed by Kimura *et al* (1996). The first primer pair was 5'-ATCATGGCTGCTTCACAGAC- and 5'-GGCTGGAGAGGACAAGACAT-3' with a product fragment of 982 bp. Nested-step PCR was conducted when negative result is found from the first-step PCR using the following primer pairs 5'-TCTTCATCAGATGCTACTGC- 3' and 5'-TAACGCTATCCAGTATCACG- 3' which amplified a 570 bp fragment. The PCR products were detected by electrophoresis of 10 µL of the reaction solution in 1.5% agarose gel for 30 minutes. This was stained in 1 µg/mL ethidium bromide solution for 5 minutes and de-stained in DDW for 10 minutes. After destaining, the gel was viewed under UV trans-illuminator (Syngene) to check DNA amplification bands positive for WSSV.

Rotifer resting egg collection

Rotifer resting eggs were collected from sediment samples of a shrimp farm in Leganes, Iloilo, Philippines that had a recent incident of WSSV outbreak. Following the method of Aparici *et al* (2001), deep sediment cores each measuring 10 cm in length were taken by a core sampler and placed in sterile plastic bottles for analysis. Collected sediments were dried at room temperature, sieved through a 25 µm mesh net and particles passing the sieved were collected. After sieving, three composite samples of 10 cm^3 each were taken to which 1.75M sucrose solution was added to make a 40-mL final volume. The soil-sugar mixture was centrifuged for 5 minutes (600 rpm). The resulting supernatant was decanted and placed in sterile Petri dish where rotifer resting eggs were collected using a capillary tube. Collected eggs were also checked by one-step PCR to confirm the presence of WSSV in these diapause eggs.

Destruction of rotifers resting eggs using industrial disinfectants

Three commercially available industrial disinfectants or biocides such as commercial bleach sodium hypochlorite (NaOCl), granulated calcium hypochlorite ($Ca(OCl)_2$) and formalin (H_2CO) were tested on their efficacies to destroy rotifer resting eggs at concentrations of 1, 5 and 10 mg L^{-1}.

These biocides were first diluted with sterile sea water (SSW) to obtain the desired concentration. This experiment was conducted using a 12-well micro titer plate. Five resting eggs were inoculated per well and triplicate run was performed on each treatment. After 24 hours, viability of the resting eggs was monitored and expressed as percent disruption.

Statistical analyses

Data from the non-viability experiment computed in percentages were first transformed to arcsin prior to testing for one-way analysis of variance (ANOVA). The Duncan's Multiple Range Test was conducted to test differences between means. All statistical analyses were done using SPSS 10.0.

Results and Discussion

White spot syndrome virus (WSSV) is a devastating disease threatening the sustainability and economic viability of shrimp culture. In the previous decades, collective knowledge in understanding the biology and pathogenecity of this virus on shrimp have significantly increased, but were not enough to develop a technology for the full control of WSSV outbreaks in crustacean

culture. Recently there is a growing interest in understanding the mechanism and ecological dynamics of viral survival in the aquatic ecosystem. Recurrence of viral outbreaks are common in shrimp ponds that have undergone thorough preparations that includes drying, liming and bottom soil desiccation, conditions considered unfavorable to virus survival. These observations led to a hypothesis of the existence of a non-crustacean host that may serve as a reservoir of WSSV in an aquatic environment. However, information on other organisms, serving as a host for WSSV had been few.

Recent works indicate that rotifers, a common constituent of the shrimp pond zooplankton community could be a host and a reservoir of WSSV. The rotifer, *Brachiounus urcerus* and their resting eggs collected from sediments of a shrimp farm stricken with WSSV in China were found to be positive with WSSV (Yan *et al* 2004). Results of the present study are in agreement with these findings. In the present study, WSSV syndrome virus was detected in the rotifer, *Brachiounus plicatilis* after exposure to tissue homogenates of WSSV infected shrimp. Further, diapause eggs of this rotifer species were also found to harbor WSSV, confirming the previous evidences that rotifers is a host and a vector in the transmission of WSSV. Additional evidence was also shown that rotifer cellular membrane contains receptors for the binding of WSSV (Yan *et al* 2007b). Binding to cellular receptors is the initial step in the process of viral entry to the host cell.

Transmission and infectivity of WSSV from infected rotifers to a crustacean hosts were documented by Yan *et al* (2007) and Zhang *et al* (2006). The work of Zhang *et al* (2006) demonstrated that WSSV from the rotifer, *Brachionus urcerus* is highly infective but were not pathogenic to the larvae of *Penaeus chinensis*. In the present study, WSSV transmitted from infected rotifers, *Brachionus plicatilis* to post-larval *P. monodon* were found to be highly pathogenic. These contrasting findings are not yet fully understood, but this could be due to the differences in the species of rotifer host, species of shrimp and the different shrimp larval stages used in these experiments. To the best of our knowledge, the present study is the first to document the high pathogenicity of WSSV transmitted from rotifers to *P. monodon* post larvae.

Rotifers immersed in a WSSV inoculum prior to feeding to shrimp (PL 21) showed higher cumulative mortalities (81% in test as opposed to 45% in negative control) than shrimp fed with uninfected rotifer (Figure 1). While this was not shown in the first-step PCR, a further test using nested PCR analysis showed that rotifers exposed to the WSSV inoculum and shrimp larvae fed with the infected rotifers were WSSV-positive (Figure 2).

One-step PCR detected WSSV in pre-disinfected samples showed that 4 out of 9 replicates were WSSV-positive with a band size of 211 base pairs (Figure 3). Detection of WSSV using one-step PCR of pooled samples indicates the high titer of the virus in the resting egg samples. As pooled samples were used, one or more of the 9 resting eggs used may be WSSV-positive.

Surface disinfection was initially used to further verify whether the virus is also present within the egg or just along the surface. However, disinfectants used at high concentrations in this study were found to make the eggs non-viable. All three disinfectants or biocides tested were effective in destroying the rotifer resting eggs after 24 hours (Figure 4).

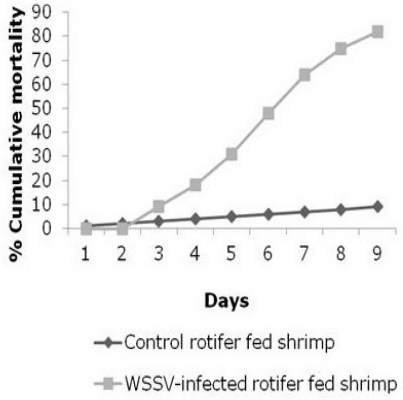

Figure 1. Mean cumulative mortality per day of penaeid shrimp post larvae exposed to WSSV fed with WSSV negative or positive rotifers *Brachionus plicatillis*

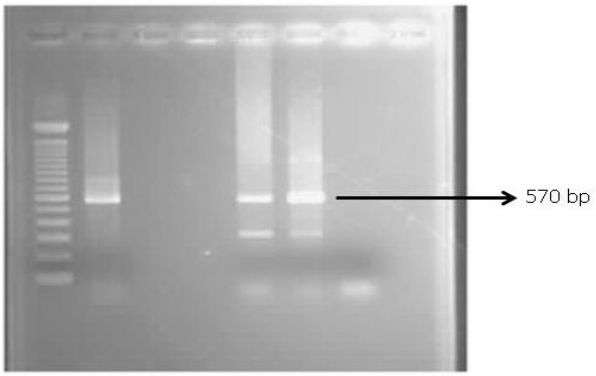

Figure 2. Detection of WSSV in hatched rotifer samples and shrimp postlarvae (PL) by two-step PCR result: M, Marker; Lane 1, positive control; Lane 2 rotifer (negative); Lane 3, shrimp (negative); Lane 4, rotifer (positive); Lane 5, shrimp (positive); Lane 6, negative control

Highest percentage (100%) of disruption was obtained by using granulated chlorine at higher concentrations (5 and 10 mg L^{-1}). However, this was not significantly different if bleach solutions at 5 mg L^{-1} (96%) and 10 mg L^{-1} (86%) were used. On the other hand, the percentage of resting eggs disruption by formalin at 5 to 10 mg L^{-1} was comparable with that of bleach at all test concentrations. At 1 mg L^{-1} of formalin, lowest percentage of disruption was observed.

Chlorine, commonly applied as sodium hypochlorite and granulated chlorine, is one of the arthropod pesticides along with trichlorophon that is used extensively for the control of zooplanktons in shrimp farms (Funge-Smith & Briggs 1998; Schuur 2003). At dosages as low as 1 ppm, it can significantly increase the hatching rate of *B. plicatilis* resting eggs after 1 hour of exposure due its action on the cyst covering and the elimination of bacterial contaminants (Balompapueng *et al* 1997). However, at higher concentrations, it renders the resting egg non-viable due to oxidation of live tissue (Gray *et al* 2006). For *Artemia sp.*, 90% of the isolated cysts became non-viable after exposure to 53 ppm of hypochlorite solution for 24 hours (Sano *et al* 2004) while 500 to 1000 ppm was required to prevent hatching of the diapausing invertebrate eggs in a non-ballasted ship (Gray *et al* 2006).

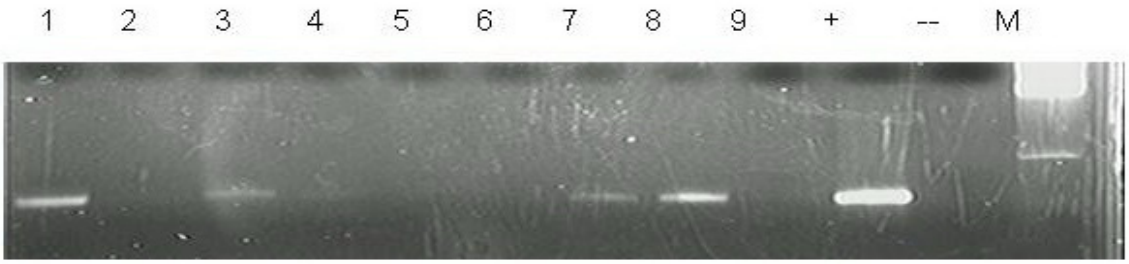

Figure 3. Detection of WSSV in pre-disinfected samples containing 9 resting eggs by one-step PCR result: Lane 1-9, rotifer samples, +, positive control; -, negative control; M, marker

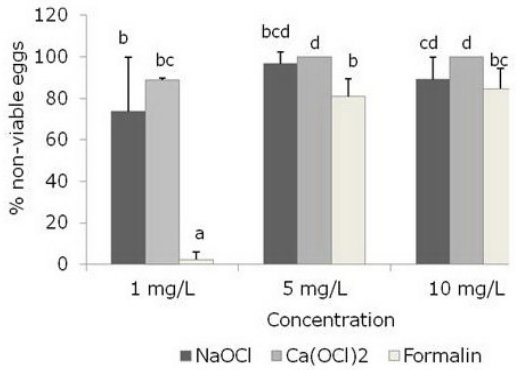

Figure 4. Mean non-viable resting (*Brachionus plicalitis*) eggs after 24 h treatments with three disinfectants

In this study, the range of effective concentration (5-10 mg/L) obtained was lower but was within the range (1-30 mg/L) of dosages typically used in the pre-stocking treatment of shrimp farms (Boyd 1995; Gräslund & Bengtsson 2001). The different effects of the high dosage concentrations on the resting eggs can be attributed to the methods used in the isolation process. Generally, the presence of sediments would require higher concentration of hypochlorite than just the isolated resting eggs as the sediments present a physical barrier to the action of the disinfectant. It also generates a high chlorine demand (Sano *et al* 2004). Hence, results of this study may underestimate the effective dose that is applicable in farm conditions. At the farm scale, its efficacy may not be replicated as sediments present numerous matrices where the resting eggs may reside; hence, decreasing the contact between the disinfectant and the eggs. Further tests using this disinfectant along with other management practices must be done in the field to be able to obtain relative success in its application.

Conclusion

Results of this study suggest that the rotifer, *Brachionus plicatilis* is a vector for the transmission of WSSV in *P. monodon* and the WSSV from infected rotifers is highly pathogenic to post-larval stage of this shrimp. Also, commercially available industrial disinfectants already used to disinfect shrimp ponds can control the resting egg stage of the rotifers, thus reducing the risk of WSSV outbreak. However, proper disinfection strategies in shrimp farms should also include pond sediments and more studies are recommended in this area.

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