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Detection of white spot syndrome virus in filtered shrimp-farm water fractions and experimental evaluation of its infectivity in *Penaeus* (*Litopenaeus*) *vannamei*

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ABSTRACT

White spot syndrome virus (WSSV) may spread through water to neighbor ponds or farms. Routine water exchange and wastewater released during white spot disease (WSD)-emergency harvests may preserve WSSV in shrimp farming areas. To test this hypothesis, on-site experiments were performed in a WSSV-affected farm in Guasave, Sinaloa, Mexico. Plankton and shrimp hemolymph were collected from 12 ponds during a WSD outbreak. PCR analyses showed that 72% of the hemolymph pools (26 out of 36) were WSSV-positive. In contrast, only 14% (4 of 28) plankton samples (filtered through 10 and 0.45 μm) from three ponds (2, 7 and 10) were WSSV-positive. Plankton from pond 9 was WSSV-negative, but 14 days later, shrimp began to die. At this point, a differential filtration experiment was performed in pond 9. WSSV-positive samples were only found in three fractions [particulate fraction (PF) 1 μm and liquid fractions (LF) <100 and <40 μm]. Both LFs and PFs were used for *in situ* infectivity assays by water-borne routes in WSSV-negative whiteleg shrimp *Penaeus* (*Litopenaeus*) *vannamei*. Some shrimp exposed to different PFs and LFs (100 μm to >0.65 μm) became WSSV-positive. Results indicate that water fractions between 100 and 0.65 μm induced WSSV infection to shrimp. Results showed that pond water and/or particulate fractions are vehicles for WSSV dispersion via virus suspended in water, attached to microalgae, or carried by zooplankton.

1. Introduction

White spot syndrome virus (WSSV) is a pathogen that has devastated the shrimp farming industry in several countries (Lightner et al., 1998; Jiang et al., 2006); currently it is the most serious shrimp viral pathogen in the world (Soto and Lotz, 2001; Flegel, 2006; Sánchez-Martínez et al., 2007). In Mexico, WSSV was first reported in 1999 and soon caused severe losses, first to the culturing of *Penaeus* (also called *Litopenaeus*) *stilyrostris* and later to *P. vannamei* (Galavíz-Silva et al., 2004; Peinado-Guevara and López-Meyer, 2006).

WSSV has a bacilliform shape and is a non-occluded, enveloped virus (Chou et al., 1995; Wongteerasupaya et al., 1995). Intact enveloped virions range from 210–380 nm in length and 70–167 nm in maximum width (Flegel and Alday-Sanz, 1998; Park et al., 1998; Rajendran et al., 1999; Escobedo-Bonilla et al., 2008). Under culturable conditions, this virus may cause up to 100% cumulative mortality in 2–10 days after the onset of symptoms (Lightner, 1996; Xu et al., 2006).

At present, no treatments are available to control the disease and mortality. The only alternative to reduce the risk of WSSV entry into commercial shrimp production facilities is the implementation of biosecurity or exclusion measures, such as filtration and disinfection (Clifford, 1999; Lightner, 2005). Water probably is a major pathway for WSSV entry into an aquaculture facility (Lotz and Lightner, 1999; Cohen et al., 2005; Corsin et al., 2005). Currently in shrimp farming, pond wastewater is routinely discharged into the adjacent environment (coastal lagoons or estuaries), where other crustacean species dwell. Many crustaceans are potentially susceptible to WSSV infection (see Escobedo-Bonilla et al., 2008). Heavy water exchange is normally done even in ponds affected by WSSV outbreaks. These practices probably increase the risk of WSSV transmission to neighboring shrimp farms.

Continuous and strict monitoring of the various components of shrimp farming is required to reduce the spread of WSSV within a region and to avoid introduction of the pathogen into a new area. These components include brooders (Hossain et al., 2004), fry, live

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feed (Lo et al., 1998; Pongmaneerat et al., 2001; Hameed et al., 2002), incoming and outgoing water (Clifford, 1999; Corsin et al., 2005). Effective monitoring should be done with highly sensitive and specific detection methods for WSSV. Several techniques have been used to detect WSSV. PCR is one of the most sensitive and specific methods available. PCR has been widely used to detect WSSV in farmed shrimp, wild and laboratory postlarvae, carrier animals, water, and sediments (Lightner, 1996, Hossain et al., 2001, 2004; Hameed et al., 2005; Natividad et al., 2008). Strict monitoring of shrimp health and other preventive measures, such as disinfection and filtration of inflowing water (Lawrence et al., 2001) can reduce the impact of WSSV.

WSSV has been widely reported in cultured and wild susceptible animals, such as shrimps, crabs, and other arthropods (Lo et al., 1996; Chakraborty et al., 2002; Hossain et al., 2004; Maldonado et al., 2004; Lightner, 2005; De la Rosa-Vélez and Bonami, 2006; Sánchez-Martínez et al., 2007; Escobedo-Bonilla et al., 2008). Possible routes for WSSV transmission include cannibalism of moribund shrimp (Wu et al., 2001; Lotz and Soto 2002), vertical transmission from infected spawners to stocked postlarvae (Sánchez-Martínez et al., 2007), and horizontal transmission from batches of infected postlarvae in a pond and subsequent spread of the pathogen to a neighboring pond or even to another farm (Wu et al., 2001; Lightner, 2005). Conversely, little work has been done to determine the presence of WSSV in shrimp-farm water and no protocols for WSSV detection in water have been described. Further, PCR detection of WSSV in water does not necessarily imply that it is infectious. Corsin et al. (2005) detected the presence of WSSV by PCR in plankton samples of ponds, but they found no association with the occurrence of WSD outbreaks. So far, no studies have assessed the potential risk of WSSV infection through shrimp-farm water. Hence, an in-farm bioassay procedure is required to determine whether pond water (raw or a specific fraction) containing WSSV is sufficient to induce infection in shrimp. Such

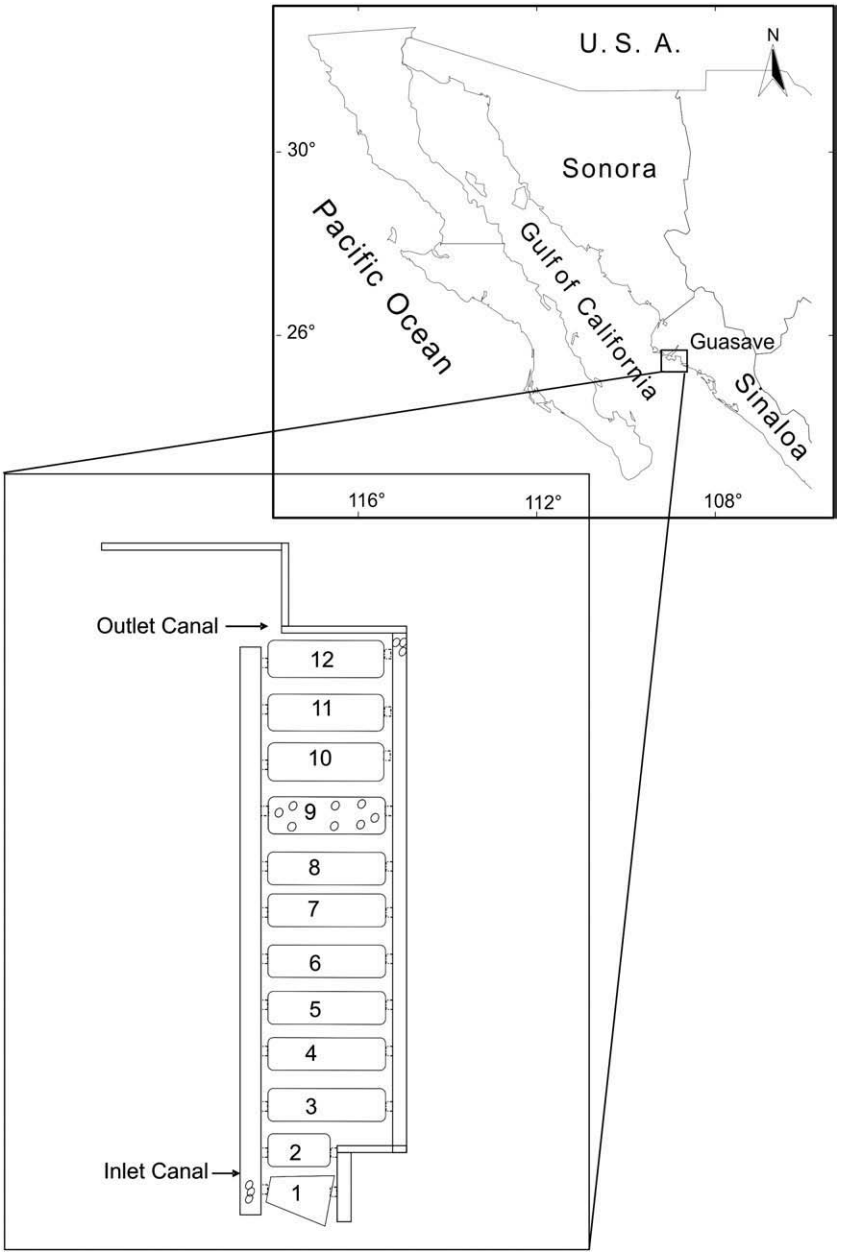

Fig. 1. Location of the shrimp farm and the pond sampling sites. Circles inside the ponds represent shrimp and water sampling points. All numbered ponds were sampled in the same manner.

data can help to understand the mechanisms for WSSV spread in ponds and the relationship between the presence of water-borne WSSV and the potential risk of a WSSV outbreak.

The objectives of this study were: (1) to develop a differential pond water filtration protocol to determine the water fraction(s) in which WSSV is found, and (2) perform an in-farm bioassay protocol to evaluate whether WSSV-positive water fractions are able to cause infection to WSSV-negative *P. vannamei* shrimp.

2. Material and methods

2.1. Study area

This study was performed at a shrimp farm located in Guasave, Sinaloa, Mexico (25° 26′ 13.23″ N, 108° 40′ 45.89″ W) (Fig. 1). The farm has 12 ponds of different sizes (2.5, 1.3, 4.0, 4.0, 5.0, 5.0, 5.0, 5.0, 5.0, 4.5, 4.5, and 4.5 ha; ponds 1 to 12, respectively). Each pond was stocked with 9 shrimp m^{-2}. Shrimp culture started on 1 August and completed on 5 November 2007. Each 12 pond was sampled once for shrimp and plankton between 21 September and 4 October. Outbreaks of white spot disease (WSD) occurred at the farm between 7 September and 20 October 2007.

2.2. Hemolymph sampling

Pooled hemolymph samples from 10 shrimp (50 µL per shrimp) were collected with a syringe containing 500 µL of anticoagulant SIC–EDTA (EDTA 20 mM, KCl 10 mM, NaCl 450 mM, HEPES 10 mM) (Vargas-Albores et al., 1993) and placed in a 1.5 mL tube. Three pools were obtained from each of the 12 ponds. Only one wild shrimp *P. vannamei* was collected from the outlet canal and it was also analyzed. Samples were transported to the Laboratory of Molecular Biology at CIIDIR–IPN in Sinaloa and immediately processed for WSSV detection.

2.3. Plankton sampling

To detect WSSV in pond water plankton, a single sampling was done in each pond as follows: one water sample (16 L) was collected from each of eight points around the shore (see Fig. 1). Water (128 L) was mixed and filtered through a 10 µm nylon monofilament-bag filter (Aquatic Eco-Systems, Apopka, FL). Plankton fractions > 10 µm were trapped in the bag, washed with 96% ethanol to concentrate the particles and placed into 1.5-mL tubes containing 96% ethanol. Afterwards, an aliquot of the remaining pond water was filtered through a 0.45 µm membrane (Durapore®, Millipore, Billerica, MA) until saturation. Membranes were individually placed into 1.5-mL tubes containing 96% ethanol. Per pond, one plankton sample filtered at 10 µm and 0.45 µm, was respectively obtained from each of the 12 ponds, ($n = 24$). Additionally, plankton samples (10 and 0.45 µm) were taken from the inlet and outlet canals, respectively ($n = 4$). A total of 28 plankton samples were analyzed by PCR at the Laboratory of Molecular Biology at CIIDIR–IPN in Sinaloa.

2.4. Differential filtration system

Pond 9 had WSSV-positive shrimp, as did other ponds. However, shrimp in this pond started to die during the sampling period. For this reason, we decided to use water from this pond to perform a differential filtration assay (see Figs. 1 and 2), as follows: Pond water (2000 L) was collected from eight sites around the pond (250 L $site^{-1}$) and mixed in the transportation tanks. First, water was filtered through a 100-µm pore-size nylon monofilament bag (Aquatic Eco-Systems) connected to a submersible pump [128.7 L min^{-1}, 1/3 horse-power (hp) Ecovort Series, Weston, FL] and placed in a 500-L tank. Particles trapped in the filter were kept in artificial seawater (Aquarium Systems Instant Ocean, Ohio, WI) at the same salinity as pond water (35 g L^{-1}). Filtered water was then serially passed through a system of 40, 20, 10, 5, and 1-µm pore-size nylon monofilament bags (Aquatic Eco-Systems) separately

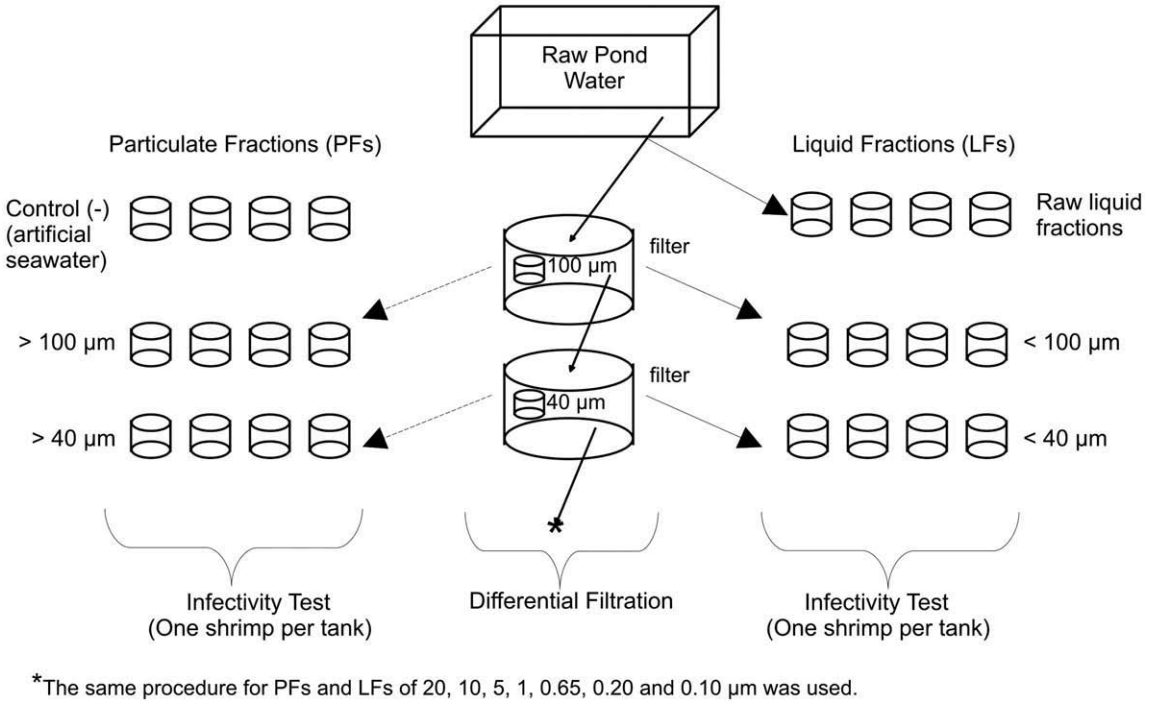

Fig. 2. Diagram of the differential filtration system. Raw pond water was filtered through a series of filter bags or cartridges with different pore sizes (100, 40, 20, 10, 5, 1, 0.65, 0.20, and 0.10 µm) to exclude WSSV, to determine the liquid (LFs) and/or particulate (PFs) fractions that contains WSSV, and whether these fractions were able to cause infection to WSSV-negative shrimp *Penaeus vannamei*.

contained in 500-L tanks. Each filtered water fraction and its respective particulate fraction was maintained in their respective 500-L tank. Water filtered through the 1-μm bag was serially passed through a 0.65, 0.20, and 0.10-μm hydrophilic cartridge filters (Durapore®, Millipore) until saturation. The filtered liquid fractions and the particulate fractions retained in the filters were used for *in situ* infectivity assays by the water-borne route (Fig. 2). Aliquots from each of the fractions were used to detect WSSV DNA by PCR analyses in duplicate.

2.5. Experimental animals

WSSV-negative shrimp *P. vannamei* (mean body weight = 7.5 ± 0.7 g) from a shrimp hatchery in Sonora, Mexico were used. Shrimp were maintained at the facilities of the Laboratory for Crustaceans, CIIDIR–IPN in Sinaloa. All the animals used in the experiments were previously tested for WSSV using nested PCR, as described below. During the acclimation and experimental periods, shrimp were fed with a commercial feed twice daily.

2.6. Experimental infectivity tests with liquid fractions and particulate fractions

Field experiments were performed inside a controlled-temperature chamber (water temperature 27 ± 0.7 °C). This temperature allows optimal WSSV replication (Rahman et al., 2006). Six HOBO (Hobo® multi-channel re-usable data loggers, Onset Computer Corporation, Bourne, MA) thermographs were installed inside experimental units to record temperature. The filtered water fractions (raw pond water and filtered through 100, 40, 20, 10, 5, 1, 0.65, 0.20, or 0.10 μm) were poured (10 L each) onto four 19-L tanks (Fig. 2). Each of the different particulate fractions was suspended in 40 L artificial seawater (35 g L^{-1}) and poured (10 L each) onto four 19-L tanks (Fig. 2). Control tanks ($n = 4$) were filled with 10 L artificial seawater (35 g L^{-1}).

In each tank, one shrimp was placed to prevent cross-infection through cannibalism. Each tank was partly sealed and under positive oxygen pressure. Pulses of pure oxygen (O_2) (10 min every three hours) were injected into each tank through an air stone.

WSSV infection was determined by nested PCR from hemolymph (50 μL) individually collected from all experimental shrimp at 0, 120, and 216 h after exposure. After each sampling, shrimp were returned to their respective tanks.

2.7. Nucleic acid extraction

2.7.1. Hemolymph

Pooled hemolymph was centrifuged (10,000 *g* for 10 min) to obtain a cellular pellet that was used for DNA extraction with DNAzol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

2.7.2. Water and plankton

Samples from the liquid fractions, particulate fractions, and filters (0.45 μm) were individually crushed with a pestle in 300 μL extraction buffer (DNAzol, Invitrogen). Samples were centrifuged (10,000 *g* for 10 min) and the supernatant was collected. In all cases, DNA extraction used the DNAzol kit, following the manufacturer's instructions. All extracted DNA was stored at −20 °C until used for PCR.

2.7.3. Origin and purity of WSSV DNA used as positive control

Shrimp *P. vannamei* were collected in farms undergoing a WSSV outbreak in Guasave Sinaloa. Total DNA was extracted (DNAzol) from tissues (0.05–0.10 g) of the collected animals and analyzed by PCR to detect WSSV and IHHNV DNA. Animals only infected with WSSV were used to prepare a WSSV inoculum. The inoculum was used to challenge WSSV-negative animals under experimental conditions. All challenged animals displayed signs of WSSV infection thus proving the presence of infectious WSSV particles (not shown). An aliquot (100 μL) of the WSSV inoculum was used for DNA extraction. The resulting WSSV DNA was used as the positive control for PCR analyses.

2.8. PCR analysis

WSSV detection was performed by nested PCR, using the primers reported by Kimura et al. (1996) (WSSVout-1/WSSVout-2 and WSSVin-1/WSSVin-2). A positive control (described above), a negative control (sterile H_2O), and an internal control (which amplified a 298 bp segment of shrimp GAPDH DNA by one-step PCR) were included. First, a one-step PCR was done with primers WSSV out-1/out-2 that produced a 982 bp amplicon from the WSSV genome. Nested PCR was done by adding 1 μL of the first PCR reaction as the target DNA, using primers WSSV in-1/in-2. These primers amplified a genome fragment of 570 bp (Peinado-Guevara and López-Meyer, 2006). Both the one-step- and nested-PCR assays were done in a PCR thermocycler (Biorad, Hercules, CA), using the following cycle parameters: initial denaturation (94 °C for 4 min) and 35 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s), extension (72 °C for 90 s), and a final extension (72 °C for 5 min).

Total reaction volumes for one-step- and nested-PCR assays were 25 μL. Reaction mix for each PCR assay contained dNTPs (0.2 mM) (Promega, Madison, WI), $MgCl_2$ (2 mM) (Invitrogen), WSSV primers (WSSV out-1/out-2) (0.5 μM each primer) (Sigma, St. Louis, MO), *Taq* DNA polymerase (1.25 U) (Invitrogen) and 1 μL template DNA or the first PCR amplicon, respectively. Afterwards, PCR products were resolved by 1% agarose gel electrophoresis, stained with ethidium bromide, observed under UV light, and recorded. The product from the nested PCR amplification was purified, concentrated, and sent to a commercial laboratory for DNA sequencing.

3. Results

3.1. WSSV status of farmed shrimp

Hemolymph collected from farmed shrimp showed that 26 of 36 pools (72%) were WSSV-positive either by one-step- or nested-PCR (Table 1). The wild shrimp collected in the outlet canal was also WSSV-positive by nested PCR. Pooled hemolymph collected from ponds 1 and 4 were WSSV-negative by one-step PCR but 2 of 3 samples (pond 1) and 1 of 3 pools (pond 4) were WSSV-positive by nested PCR. In contrast, all pooled samples from ponds 9 and 11 were

Table 1
WSSV PCR analyses (one-step or nested PCR) in pooled hemolymph samples from shrimp collected in ponds and outlet canal.

Pond	Number of pools examined[a]	Number of WSSV-positive pools	
		One-step PCR	Nested PCR
1	3	0/3	2/3
2	3	1/3	1/3
3	3	2/3	2/3
4	3	0/3	1/3
5	3	2/3	2/3
6	3	1/3	2/3
7	3	1/3	2/3
8	3	1/3	3/3
9	3	3/3	3/3
10	3	2/3	3/3
11	3	3/3	3/3
12	3	1/3	2/3
Outlet canal[b]	1	0/1	1/1
Inlet canal	NA	NA	NA

NA = not available.
[a] Each pool corresponds to hemolymph of 10 shrimp.
[b] Only one wild shrimp *P. vannamei* was collected.

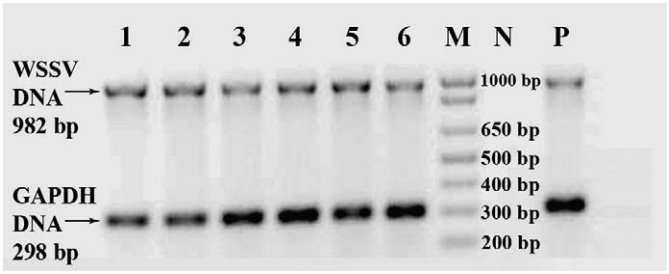

Fig. 3. WSSV detection by one-step PCR in hemolymph collected from farmed shrimp. Lanes 1–3: pooled samples from pond 9; Lanes 4–6: pooled samples from pond 11; Lane M: DNA weight marker; Lane N: Negative control; Lane P: positive control.

WSSV-positive by one-step PCR (Fig. 3). The PCR product was sequenced and it fully corresponded to the WSSV genome segments 213026 to 214007 (982 bp) from the Thailand isolate (GenBank accession no. FJ789570; Peinado-Guevara and López-Meyer, 2009).

3.2. WSSV in pond water and plankton

A total of 28 plankton fractions (12 plankton fractions>10 μm and 12 plankton fractions>0.45 μm) were collected from the 12 ponds ($n=24$) and the inlet and outlet canals ($n=4$). Of these, only four samples from ponds 2, 7, and 10 were WSSV-positive by nested PCR. Only a plankton fraction >10 μm was found WSSV-positive in pond 7, whereas WSSV-positive plankton fractions >0.45 μm were found in ponds 2, 7, and 10. Plankton fractions from the inlet and outlet canals were WSSV-negative. The PCR product was sequenced and it fully corresponded to the WSSV genome 213218 to 213787 (570 bp) from the Thailand isolate (GenBank accession no. FJ609650).

3.3. Differential filtration assay and infection test

The differential filtration assay showed that water from pond 9 was WSSV-positive by nested PCR in the liquid fraction (<100 and <40 μm) and particulate fraction (<1 μm) 14 d after initial sampling of the pond water (Fig. 4). Raw pond water was WSSV-negative. The presence of WSSV DNA in these fractions was not consistent for all replicates (Table 2).

The infectivity assay showed that only four particulate and three liquid fractions caused WSSV infection to exposed shrimp. In the particulate fraction >1 μm, one WSSV-positive shrimp was first detected at 120 h after exposure. At 216 h after exposure, this same animal and five others in the >100, >40, and >5 μm particulate fractions were WSSV-positive (Fig. 5). In the <40, <10, and <0.65 μm liquid fractions, four WSSV-infected animals were identified up to 216 h after exposure (Table 3). WSSV-positive animals were not found in all four replicates from the same fraction. In contrast, no WSSV-positive shrimp were identified from raw liquid fractions at any time during the experiment.

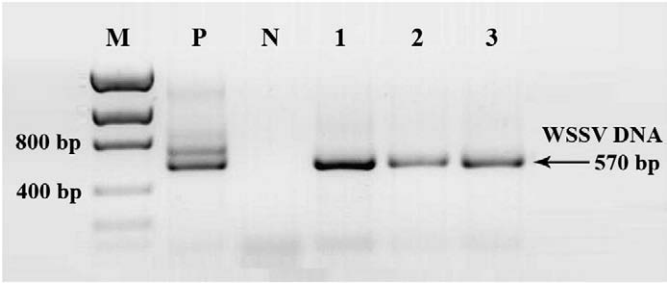

Fig. 4. WSSV detection by nested PCR in liquid (LFs) and particulate (PFs) water fractions collected from pond 9. Lane M: DNA weight marker; Lane P: positive control; Lane N: negative control; Lane 1: PF≥1 μm; Lane 2: LF≤100 μm; Lane 3: LF≤40 μm.

Table 2
Proportion of WSSV-positive particulate (PFs) and liquid (LFs) water fractions collected from pond 9.

Fraction size (μm)	Fractions type (proportion of WSSV-positive[a])	
	PFs	LFs
[Raw pond water]	–	0/2
≥100 [≤100]	0/2	1/2
≥40 [≤40]	0/2	1/2
≥20 [≤20]	0/2	0/2
≥10 [≤10]	0/2	0/2
≥5 [≤5]	0/2	0/2
≥1 [≤1]	1/2	0/2
≥0.65 [≤0.65]	0/2	0/2
≥0.20 [≤0.20]	0/2	0/2
≥0.10 [≤0.10]	0/2	0/2

[ ] = Fraction size of liquid fractions.
[a] Sampling and analyses were done in duplicate.

4. Discussion

This study showed that WSSV virions can be found in different water fractions from a pond undergoing a WSD outbreak when daily temperature ranged from 30 to 33 °C and have the potential to infect shrimp at 27 °C. These results suggest that infectious WSSV may be associated with plankton subpopulations, such as microplankton (20–200 μm), nano-plankton (2–20 μm), and/or picoplankton (0.2–2.0 μm) (see Table 3).

Different plankton components, such as microalgae, rotifers, and copepods have been mentioned as potential carriers of infectious WSSV. Experiments done with marine microalgae (*Isochrysis galbana, Skeletonema costatum*, *Chlorella* sp., *Heterosigma akashiwo, Scrippsiella trochoidea,* and *Dunaliella salina*) exposed to WSSV-infected shrimp showed that plankton served as WSSV carriers and induced infection to shrimp (Liu et al., 2007). Other studies showed that rotifers may be WSSV carriers and an overwinter reservoir for WSSV (Yan et al., 2004, 2007a). Further, it was shown that cell membranes from the rotifer *Brachionus urceus* bind WSSV in vitro (Yan et al., 2007b). Rotifers may be WSSV carriers through ingestion of WSSV-contaminated phytoplankton and become a vector in WSSV transmission to penaeid shrimp larvae (Zhang et al., 2006). Likewise, the

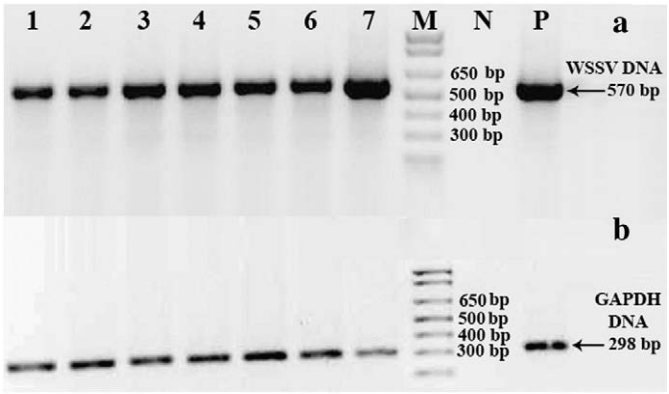

Fig. 5. (a) WSSV detection by nested PCR in shrimp exposed to different water fractions at 216 h after exposure (hae). (b) PCR amplification of the internal control. Lane 1: LF≤40; Lane 2: LF≤10; Lane 3 LF≤0.65 μm; Lane 4: PF≥100; Lane 5: PF≥40; Lane 6: PF≥5; Lane 7: PF≥1 μm. Lane M: DNA weight marker; Lane N: negative control; Lane P: positive control.

Table 3
Proportion of WSSV-positive shrimp exposed to different water fractions from pond 9 at 0, 120, or 216 h after exposure (hae).

Fraction							
Size (μm)	Liquid			Size (μm)	Particulate		
	Time (hae)				Time (hae)		
	0	120	216		0	120	216
Raw	0/4	0/4	0/4	–	–	–	–
≤100	0/4	0/4	0/4	≥100	0/4	0/4	2/4
≤40	0/4	0/4	1/4	≥40	0/4	0/4	2/4
≤20	0/4	0/4	0/4	≥20	0/4	0/4	0/4
≤10	0/4	0/4	2/4	≥10	0/4	0/4	0/4
≤5	0/4	0/4	0/4	≥5	0/4	0/4	1/4
≤1	0/4	0/4	0/4	≥1	0/4	1/4	1/4
≤0.65	0/4	0/4	1/4	≥0.65	0/4	0/4	0/4
≤0.20	0/4	0/4	0/4	≥0.20	0/4	0/4	0/4
≤0.10	0/4	0/4	0/4	≥0.10	0/4	0/4	0/4
Control[a]	0/4	0/4	0/4	Control[a]	0/4	0/4	0/4

[a] Artificial seawater.

harpacticoid copepod *Nitocra* became a WSSV vector through ingestion of WSSV-positive phytoplankton. Animals exposed to WSSV-positive herpaticoids became infected, as determined by nested-PCR (Zhang et al., 2008). These data support the notion that various phytoplankton components (micro- and nano-plankton) and zooplankton (microplankton) are WSSV carriers. It is possible that larger planktonic organisms carry more infectious WSSV particles and, when ingested by shrimp, are more likely to cause WSSV infection.

Our study showed that infectious WSSV particles were present in various water fractions from a pond undergoing a WSD outbreak, despite the fact that no viral DNA was detected by nested PCR in 0.65, 5, and 10 μm water fractions, but induced infection to some of the exposed shrimp. These results indicate that low infectious doses of WSSV may lie below the detection limits of the PCR methods used here. This situation has been observed in other studies. An in vivo titration experiment determined the virus titer of a WSSV inoculum (Escobedo-Bonilla et al., 2005). An inoculum concentration of 10^{-5} induced 80–100% mortality in shrimp by intramuscular injection. Nonetheless, the minimal concentration found WSSV-positive either by one-step- or nested-PCR was 10^{-4} (unpublished results). This finding supports the hypothesis that low infectious doses may lie below the detection limit of these PCR assays.

WSSV was not detected in experimental organisms exposed to pond water fractions <0.20 μm and artificial seawater (control). This result indicates that WSSV was not present in these test animals. It also showed that filtering water at 0.20 μm effectively excluded WSSV. Such a small pore filter size retains even free WSSV particles, as its average size is 0.27 μm (Flegel and Alday-Sanz, 1998; Park et al., 1998; Rajendran et al., 1999; Escobedo-Bonilla et al., 2008).

Raw water and shrimp exposed to raw water under experimental conditions were not WSSV-positive. Two hypotheses are proposed to explain this result: (1) the time used to incubate shrimp with raw water (216 h after exposure) may not be enough to induce infection with the infectious viral load present in raw water. We observed that WSSV-negative shrimp stocked in raw water from several ponds with WSD became WSSV-positive 552 h after exposure (unpublished data); (2) distribution of infectious WSSV in raw water or in a certain particulate or liquid fraction may not be homogeneous. Thus, high infectious doses of WSSV might only be achieved in some replicates and hence, infection will become a probabilistic issue.

Our results show that all the ponds with WSSV-positive plankton also had WSSV-positive shrimp, but not all ponds with WSSV-positive shrimp necessarily had WSSV-positive plankton (Tables 1 and 2). Moreover, a first sampling in pond 9 showed WSSV-positive shrimp and WSSV-negative plankton, but 14 days later, WSSV-positive samples were found in both shrimp and plankton (Tables 2 and 3). These field results agree with the bioassay findings, where shrimp become WSSV-infected in water fractions where WSSV was not detected. It is worth notice that pond water temperature during plankton sampling was 30–33 °C. It is known that water at 32 °C inhibits WSSV replication (Rahman et al., 2006). During this survey, partial protection by hyperthermia may have occurred to reduce WSSV replication in some ponds, which might explain the presence of WSSV-positive shrimp and WSSV-negative plankton. This result agrees with experimental data in which animals exposed for 6 h to hyperthermic conditions led to reduced mortality and inhibition of WSSV replication (Rahman et al., 2007).

One study in India showed no relationship between 10% of the ponds and WSSV-detection in plankton and WSD outbreaks (Corsin et al., 2005]). In our study, all the water fractions where WSSV was detected induced infection to shrimp at 27 °C.

In summary, this study showed WSSV DNA in various particulate and liquid fractions between 0.45 and 100 μm and WSSV particles associated with these fractions caused infection in WSSV-negative whiteleg shrimp at 27 °C. Both plankton fractions and water serve as vehicles of WSSV transmission in different ways, such as (1) Free virus released into water by WSSV-infected animals, (2) Virus associated with different phytoplankton or zooplankton species. It is possible that WSSV moves through the plankton food web as zooplankton feed on phytoplankton. Wastewater from routine exchange and emergency harvests represents a risk for spreading WSSV between farms and ponds. The current pond filtration procedures used in pond inlets (>300-μm mesh size) are inadequate to prevent WSSV from entering shrimp ponds. WSSV associated with plankton components and its movement through the plankton food web remains unclear and requires further research.

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