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Detection of white spot syndrome virus in seston from a coastal ecosystem and a shrimp farm in the Gulf of California

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Abstract
Three molecular assays were used to detect and quantify white spot syndrome virus (WSSV) in DNA extracted from seston size-fractioned (0.02, 0.2, 1.2, and 20 µm) samples collected from a coastal lagoon and an adjacent shrimp farm. From 107 DNA extracts, only two from one sample tested positive for WSSV with nested PCR in the 1.2 and 20 µm fractions. These results were confirmed by a semi-quantitative (IQ_{2000}^{TM} WSSV Detection and Prevention System) and a quantitative (IQ_{REAL}^{TM} WSSV Quantitative System) detection system based, based, respectively, on nested PCR and real-time PCR. A first viral load reference value (6.54×10^4 WSSV copies/mL) was established in a seston size fraction (1.2−20 µm). The results suggest that WSSV could be associated with both resuspension of fine clays and silts, and nanoplankton and organic colloids during infectious events.

Keywords: WSSV; seston size-fractions; nested PCR; real-time PCR; Macapule lagoon.

Detección del virus del síndrome de la mancha blanca en un ecosistema costero y una granja de camarón en el Golfo de California

Resumen
Con el fin de detectar y cuantificar el virus del síndrome de las manchas blancas (WSSV) en extractos de ADN de muestras de seston fraccionadas por tamaño (0.02, 0.2, 1.2 y 20 µm) de una laguna costera y una granja camaronícola contigua se emplearon tres ensayos moleculares. De 107 extractos de ADN, solo dos de una muestra resultaron con detección positiva al WSSV con PCR anidada en las fracciones de 1.2 y 20 µm. Estos resultados fueron confirmados por un sistema de detección semicuantitativo (IQ_{2000}^{TM} WSSV) y uno cuantitativo (IQ_{REAL}^{TM} WSSV) basados, respectivamente, en PCR anidada y en PCR en tiempo real. Se estableció un primer valor de referencia de carga viral (6.54×10^4 copias WSSV/mL) en una fracción de tamaño de seston (1.2–20µm). Los resultados sugieren que, durante eventos infecciosos, el WSSV podría estar asociado tanto a la resuspensión de arcillas y limos finos, como a nanoplancton y coloides orgánicos.

Palabras clave: WSSV; fracciones por tamaño de seston; PCR anidada; PCR en tiempo real; Laguna de Macapule.

1 Introduction

Manuscript The White Spot Syndrome Virus (WSSV) is currently regarded as one of the most virulent pathogens affecting commercial farming of various shrimp species and other marine crustaceans [1]. After being detected for the first time in China in 1992, its outbreaks were observed two years later in Texas [2] and four years later in Panama, Honduras, and Guatemala. Shrimp farms in Mexico and other Latin

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American countries have suffered substantial economic losses due to this virus [3]. Most efforts to address this disease have focused on developing early detection protocols, identifying potential transmission vectors [4], and, more recently, preventing outbreaks (e.g., [5]).

Some studies in Mexico have identified mechanisms through which the virus enters or remains latent in the farming facility system [6]. However, the possibility of the virus being present in the surrounding coastal environment and entering shrimp farms with seawater fed into reservoirs and ponds has been poorly assessed in the Mexican region. This hypothesis has been partially validated in several regions by the positive detection of WSSV in wild animals [7-10] by experimental tests. These evaluated viral infectivity through contact, ingestion of infected zooplankton (e.g., polychaetes, Artemia, copepods, and rotifers), or by virus attached to phytoplankton exopolymers that had been near sick or dying organisms [8,11,12]. Planktonic microorganisms and other seston elements are not completely filtered out of the incoming seawater stream; thus, this could be an entry pathway for disease-causing vectors into the ponds.

Even though annual technical reports of the Local Aquaculture Health Boards (JLSA, for its acronym in Spanish) of the state of Sinaloa show fewer WSSV infectious outbreaks occurring in 2019 and 2020 (27 and 8 cases, respectively) than those registered in previous periods between 2006 and 2010 (range: 115 and 273), the incidence of positive cases in shrimp farms is still currently high, mainly throughout the first and the most crucial cycle (up to 355 farms with outbreaks) of the three annual production cycles [13-15]. Therefore, time series recording and retrospective analysis of indicators of potential WSSV carriers can, even today, be of great use in local farming systems to understand patterns and address the contagion problems prevalent to date.

The present study aims to provide local evidence of the potential route of WSSV transport between shrimp ponds and its contiguous coastal system from the northern State of Sinaloa, Mexico, over a time series of viral detections on size-fractionated seston samples collected during an infectious event in June 2007 and from December 2007 to December 2008, analyzed through molecular testing based on nested, and real-time PCR assays. Viral concentration, in terms of WSSV copy number per milliliter, was estimated in the positive DNA extracts from the filtered seawater samples as an indicator of the detection limit, i.e., the minimum number of viral DNA copies in seawater detected with the methods used.

2 Material and methods

2.1 Sampling of seawater and shrimp pond water

Water samples were collected at two sites (reservoir and pond) within the Finca Doña Luisa S. de R.L. de C.V. shrimp farm and two sites (northern mouth and El Tortugón estuary) in the Macapule lagoon. The lagoon sampling stations were separated ~9 km from each other and 2.1 km and 10.8 km from the shrimp farm, respectively (Fig. 1).

Water samples between 4 and 10 L at the lagoon were collected monthly from December 2007 to December 2008 using a segmented tube; ~1 L samples at the farming facility were collected manually from the surface (0.5 m depth) in June 2007, and monthly from March to November 2008.

2.2 Seston separation by size fractions and DNA extraction

Samples were sieved through a 200 µm-mesh net and then separated into size fractions using a series of graded filters: Nuclepore polycarbonate membranes (20 and 0.2 µm), GF/C glass microfiber filters (1.2 µm), and Anodisc aluminum oxide membrane filter (0.02 µm). The volume of seawater filtered ranged from 20 mL for the smaller pore sizes to 1000 mL for the larger ones.

DNA was extracted from seston retained in the filters using DNAzol™ reagent (Invitrogen™), following the manufacturer's instructions; a total of 107 DNA extractions were obtained.

2.3 Polymerase Chain Reaction (PCR) and documentation

To assess the quality of DNA extracts, we amplified 16S rDNA with the universal primers F2C (5′-AGAGTTTGATCATGGCTC-3′) and C (5′-ACGGGCGGTGTGTAC-3′) in those availed extracts from both lagoon and pond, collected from December 2007 to July 2008 [16]. PCR amplification thermal conditions consisted of 94 °C for 4 min (initial denaturation) and 32 cycles at 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing), 72 °C for 2 min (extension), and 72 °C for 5 min (final extension). The WSSV was initially amplified using a standard nested PCR assay [17], following the thermal conditions at 94 °C for 4 min (initial denaturation), 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), 72 °C for 1 min (extension), and 72 °C for 5 min (final extension), with 30 and 35 cycles for the one-step- and nested-PCR assays, respectively, from denaturation to extension step. These amplifications were confirmed with two commercial kits, namely IQ_{2000}^{TM} WSSV Detection and Prevention System (based on nested PCR assay) and IQ_{REAL}^{TM} WSSV Quantitative System (based on real-time PCR assay) following the manufacturer's instructions (GeneReach Biotechnology Corp.).

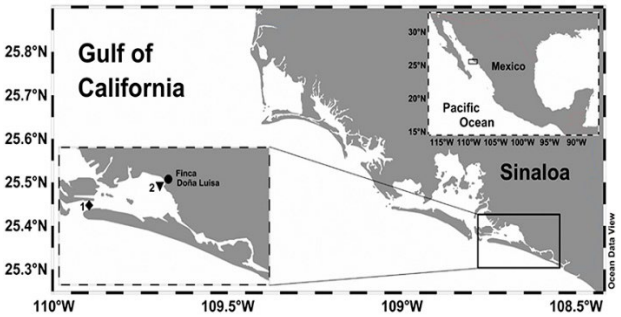


Figure 1. Location of the sampling stations at the Macapule Lagoon (Station 1: northern mouth, 25.37° N, 108.74° W, Station 2: El Tortugón estuary, 25.39° N, 108.67° W) and Finca Doña Luisa shrimp farm (closed circle). Source: The authors.

All amplified products from IQ_{2000}^{TM} WSSV Detection and Prevention System were mixed with a loading buffer [0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol F.F., 30 % glycerol in distilled water], separated by electrophoresis on 1 % agarose gel stained with ethidium bromide (0.5 mg/mL) in 0.5X T.B.E. buffer at 70 V, and then visualized using a U.V. trans-illuminator. A 1 kb molecular-weight size marker was loaded into the gel and the PCR products to identify the approximate size of the amplified fragments. Based on the viral load found in positive samples amplified with the IQ_{2000}^{TM} WSSV protocol, three possible migration patterns were expected on the agarose gel: three (910, 550, and 296 bp), two (550 and 296 bp), or a single (296 bp) band, which would indicate a high (>2000 copies/reaction), moderate (>200–2000 copies/reaction), or low (>20–200 copies/reaction) WSSV concentration, respectively. WSSV copies in the TaqMan IQ_{REAL}^{TM} WSSV assay products were quantified (absolute values) with the equation generated from the threshold cycle that relates Cq values to the log template amount. The viral concentration in the WSSV-positive extracts, as viral copies per volume (mL), was estimated by considering the filtered volume of seawater for DNA extraction.

3 Results and discussion

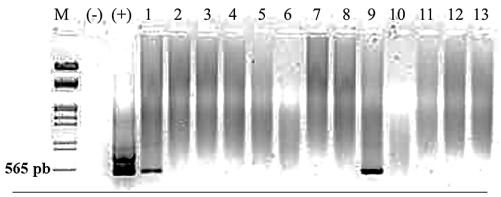
3.1 WSSV detection in seston size fractions

Only two (1.2 and 20 μm fractions collected in the shrimp pond in June 2007 and July 2008) of the 107 DNA extracts tested with the standard nested PCR assay proposed by Kimura *et al.* (1996) were positive for WSSV (Fig. 2). Both nested PCR results were confirmed by IQ_{2000}^{TM} WSSV diagnostic kits, but the IQ_{REAL}^{TM} assay returned a WSSV negative result for the June 2007 sample. When considering the filtered water sample volume for DNA extraction in the results of this quantitative assay, a viral concentration of 6.54×10^4 WSSV copies/mL was estimated for the July 2008 sample, which is 2 to 3 orders of magnitude larger than the semiquantitative kit IQ_{2000}^{TM} WSSV (Fig. 3).

This study applied methodological procedures commonly used to diagnose WSSV in animal tissues to seston samples from a shrimp farm and natural environments. Sample storing and handling (e.g., during the removal of potential inhibitors from seawater) may have affected DNA extraction, leading to our inability to detect the virus in most samples; thus, caution is advised in interpreting whether a sample was WSSV positive or not.

High concentrations of phenolic compounds, heavy metals, humic acid, or urea are the most common agents in environmental samples interfering with PCR detection (e.g., [18-20]. Of these compounds, only urea has been found in high and variable concentrations in the water column at Macapule Lagoon [21], although the significant levels of heavy metals in soft tissues of bivalves might be a potential pathway from sediments and settling seston [22].

Assuming that prokaryote DNA is ubiquitous and more abundant than WSSV DNA in seston size fractions, the results of the DNA quality test in extracts from both Lagoon and pond samples (n = 92) showed that only 50% of them

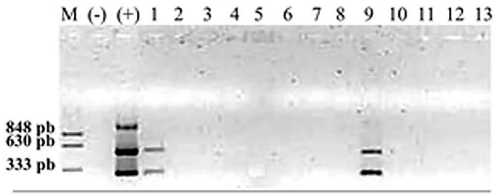


Line	Site/month	Size fraction (μm)	Nested PCR detection [17]
(-)	Negative control	*	*
(+)	Positive control	*	Positive
1	Shrimp pond (Jun-07)	1.2-20	Positive
2	Shrimp pond (Jun-07)	0.2-1.2	Negative
3	Shrimp pond (Jun-07)	20-200	Negative
4	Shrimp pond (Jun-08)	20-200	Negative
5	Shrimp pond (Jun-08)	1.2-20	Negative
6	Shrimp pond (Jun-08)	0.2-1.2	Negative
7	Shrimp pond (Jun-08)	0.02-0.2	Negative
8	Shrimp pond (Jul-08)	20-200	Negative
9	Shrimp pond (Jul-08)	1.2-20	Positive
10	Reservoir (May-08)	20-200	Negative
11	Reservoir (May-08)	1.2-20	Negative
12	Northern Mouth (Dec-08)	20-200	Negative
13	Northern Mouth (Dec-08)	1.2-20	Negative

Figure 2. Photo-documentation of nested PCR (Kimura *et al.,* 1996) results for samples from the pond and reservoir sites at Finca Doña Luisa shrimp farm and the northern mouth of the Macapule lagoon. M: 1 kb molecular-weight size marker. The band at 570 bp indicates positive WSSV detection. * Not applicable.
Source: The authors.

produced clear single bands on the agarose gel electrophoresis. Thus, DNA preservation and inhibitory components in these samples were not likely to affect the WSSV test results obtained with nested PCR. The relatively low volume of filtered seawater may have been another contributing factor in the recurrent negative detection of WSSV. However, water samples (~20 L, collected at the estuary in March 2009) that had been pre-concentrated by tangential filtration (>0.02 μm) tested negative for WSSV with nested PCR.

The WSSV positive results showed that the 1.2 and 20 μm fractions could be a potential route for transmitting this virus to crustaceans by either direct contact or ingestion by filter feeders. Inert and biological particles in this size range present in coastal lagoons mainly include resuspended sediments, self-assembled organic colloids (microgels), and nanoplankton. It is known that WSSV can remain viable in shrimp pond sediments for up to 19 days under sun-drying conditions and up to 35 days in undrained sediments [23,24].



Line	Site/month	Size fraction (µm)	Detection / extract concentration (copies• reaction^{-1})	Extrapolated concentration to sample volume (WSSV copies•ml^{-1})
(-)	Negative control	*	0 – <20	*
(+)	Positive control	*	Positive / >2000	*
1	Shrimp pond (Jun-07)	1.2-20	Positive /200-2000	~ 33-333
2	Shrimp pond (Jun-07)	0.2-1.2	Negative	
3	Shrimp pond (Jun-07)	20-200	Negative	
4	Shrimp pond (Jun-08)	20-200	Negative	
5	Shrimp pond (Jun-08)	1.2-20	Negative	
6	Shrimp pond (Jun-08)	0.2-1.2	Negative	
7	Shrimp pond (Jun-08)	0.02-0.2	Negative	
8	Shrimp pond (Jul-08)	20-200	Negative	
9	Shrimp pond (Jul-08)	1.2-20	Positive / 200-2000	~ 17-167
10	Reservoir (May-08)	20-200	Negative	
11	Reservoir (May-08)	1.2-20	Negative	

Figure 3. Photo-documentation of nested PCR results and WSSV range concentration as estimated from the IQ_{2000}^{TM} WSSV assay for some samples from the pond and reservoir sites at Finca Doña Luisa shrimp farm and the northern mouth of the Macapule lagoon. M: 1 kb molecular-weight size marker; migration bands of 848 bp, 630 bp, 333 bp. *Not applicable.
Source: The authors.

Clay and very fine silt are the dominant sediment fractions in the Macapule Lagoon [25], and their resuspension into the uppermost water layer is facilitated by wind-induced turbulence, currents, and tides flowing over the topographic features [21,26,27]. Consequently, WSSV attached to sediments may play a significant role in the outbreak dynamics when they become resuspended or, indirectly when they come into contact with eggs of planktonic organisms and invertebrates that shrimp consume [23].

Self-assembled microgels are abundant in productive systems and act as microbial activity hotspots and reservoirs of detritus, small cells, and viable viral particles [28]. Highly "sticky" fractions of such microgels, which were retained by 0.4 µm pore polycarbonate membranes and quantified with the Alcian Blue staining method, were found in high concentrations (range 0.11 to 49 µg GX eq. ml^{-1}; for methods see [29] in seawater samples from Ohuira coastal lagoon, near our study site. However, no experimental data are currently available to support the potential association between self-assembled microgels and the viability and transport of WSSV.

A wide range of planktonic organisms (e.g., microcrustaceans, rotifers, and microalgae), benthonic crustaceans, and Polychaeta worms that are not WSSV infected have been reported as carriers [30,31]. Some life stages of these organisms may be present in both plankton and benthos, increasing the probability of acquiring and spreading the disease. In shrimp farms and other Mexican coastal systems, including the Macapule lagoon, the most frequently observed potential vectors of WSSV in meso- and macro-zooplankton belong to non-crustacean groups, such as chaetognaths and fish larvae [32,33]. Esparza-Leal et al. [6] monitored 12 ponds in a shrimp farm at Guasave City for two weeks after a WSSV outbreak and found WSSV-positive results in the >10 µm and >0.45 µm plankton fractions from one (#7) and three (#2, #7 and #10) ponds, respectively. The same study conducted infectivity assays on healthy shrimp differentially exposed to ten-sized fractions (0.1 to 100 µm) of filtered water and particulate material from a pond after a WSSV outbreak. They found positive detection after 216 h in shrimp that had been exposed to the liquid fractions: <40 µm, <10 µm, and <0.65 µm; and the particulate fractions: >100 µm, >40 µm, and >5 µm. Moreover, not all replicates from the same fractions tested positive for WSSV. This variability was attributed to the relatively high temperature (30–33°C) in the experimental systems, which might have inhibited WSSV replication [34].

Nanoplankton (encompassing phyto- and zooplankton) is one of the most prolific and essential components in the planktonic food web structure in natural and artificial systems influenced by enrichment conditions. The average nanophytoplankton abundance in the Macapule lagoon and the shrimp culture system during the study period was $7.90 \pm 5.52 \times 10^3$ and $2.13 \pm 1.14 \times 10^4$ cells/mL, respectively [21,35]. The environmental conditions in ponds favor autotrophic production and microbial abundance, thus increasing the probability of encounters between nanoplankton cells and hosts releasing WSSV particles. This association partially supports the empirical model of viral transmission to higher trophic levels by virus attached to phytoplankton [8,36], although this hypothesis has not been tested in samples from natural environments.

Moreover, the possibility that coastal ecosystems function as temporary or permanent WSSV reservoirs due to the discharges of untreated pond wastewater cannot be ruled out, as reported by several investigations elsewhere [9,10,37,38]. Discharge operations are carried out during the shrimp harvesting season with no wastewater sanitization/treatment. Besides potentially affecting the local diversity of crustaceans, the virus could likely be reintroduced and infect animals in the aquaculture system during seawater supply. Moreover, post-tropical storm conditions — i.e., high temperature and high ammonia concentration, low density of heterotrophic bacteria, and low oxygen concentration — have been identified as triggers of WSSV outbreaks [5,39]. Thus, the weather might play a key role by modifying currents, precipitation, and temperature conditions, among other ambient conditions.

4 Conclusion

DNA. extraction methods should be improved to increase extraction efficiency (quantity and quality). These

improvements imply increasing filtered seawater volume by using tangential flow filtration systems to enable a higher sensitivity in determining the seston fractions involved in WSSV transmission between natural and shrimp pond ecosystems. It is also advisable to reassess the detection methodology to address the high haplotype diversity recorded, possibly due to a high viral mutation rate or the introduction of new viral strains [40]. Although advances in implementing biosecurity and contingency protocols have decreased mass mortalities produced by WSSV outbreaks in recent years, continuous monitoring and retrospective analysis of environmental and biological variables, including potential pathways of infection, is essential to discern a pattern in the recurrence of WSSV outbreaks for further prevention its spread along natural and aquaculture systems.

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