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Research Article

HIGH YIELD PURIFICATION OF VIABLE WHITE SPOT VIRUS PARTICLES FROM INFECTED *PENAEUS MONODON*

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Abstract: White spot syndrome caused by white spot syndrome virus (WSSV) is one of the most threatening diseases of shrimp culture industry. Globally, research is being carried out aiming at a better understanding on biology and pathology of WSSV and sub-sequential proper treatment and prevention. An efficient isolation and purification of viral particles is essential for these studies. This study reports a yield purification of viable and virulent WSSV virions from infected *Peneaus monodon*. The obtained yield was 8.64×10^{10} WSSV virions per 5 g infected tissue. Additionally, the yield dynamics of the complete process was studied for understanding of copy number variations. The present methodology and the yield-dynamics study of the process will lead to more improvements to WSSV purification in terms of simplicity and efficiency in the future. Moreover, the study will help in identification of the structural proteins of WSSV and shrimp bioassays to reveal the mechanisms of WSSV–host interaction.

Keywords: *Tiger shrimp, Aquaculture, Virus, Disease*
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Introduction

Shrimp aquaculture is the fastest-growing aquaculture sector and the major earning industry in many tropical and subtropical countries of the world, especially in Asia. According to FAO [1], global production of farm-raised shrimp reached 9.3 million tonnes in 2018, with *P. vannamei* contributing 52.9% to the output. However, shrimp aquaculture has been marred with certain issues including pollution, less productivity, pathogens, etc. Since 1981, several pathogens have been reported in shrimp farms causing mass mortalities and threatening the economic sustainability of the shrimp aquaculture industry. Among all the 20 viruses reported so far, white spot disease caused by white spot syndrome virus (WSSV) has had the most significant impact on shrimp culture and continues to be an obstacle to sustainable shrimp farming worldwide [2]. Since the first report in China and Taiwan in the early 1990s, the WSSV has been associated with huge economic losses in the global shrimp production industry [3,4]. Globally, the total financial losses caused by the disease to the shrimp industry have been estimated to be around USD 8-15 billion since its emergence [5] and had been increasing by USD 1 billion yearly. Many reports suggest that the annual economic losses due to WSSV are approximately one-tenth of the global shrimp production [6].

WSSV is a non-occluded enveloped, double-stranded DNA virus, ovoid to bacilliform in shape with a tail-like extension at one end [7]. It is the only member of the family Nimaviridae, genus Whispovirus and has an exceptionally broad host range being pathogenic to at least 78 species, mainly decapod crustaceans [8]. The diameter of the virions is 70-138 nm x 240-340 nm containing a rod-shaped nucleocapsid of 70-90 x 200-350 nm with genome size varying from 292 kb to 307 kb in different WSSV geographical isolates [7,9]. Infected shrimp display clinical signs such as lethargy, swollen branchiostegites due to fluid accumulation, white spots in the cuticle, loose cuticle from the underlying epidermis, yellowish-white and enlarged hepatopancreas, hemolymph failure to coagulate and reddish discoloration of the moribund shrimp [3]. The virus usually causes up to 100% mortality within 3-10 days in shrimp of all sizes from the inception of visible gross signs [3] and has been classified as a C-1 category pathogen [10].

An efficient method to purify the intact WSSV viral particles with high yield is essential for the research and study (*e.g.*, identification and characterization of structural proteins) of this virus.

In the past, mainly gradient centrifugation using sucrose or sodium bromide has been widely used [11,12]. However, the need for ultracentrifugation in these methods for virus purification is disadvantageous for intact viral particles. Additionally, abundant intact virions are difficult to be recovered using these methods, and relatively minor structural proteins are mostly unavailable for further analysis. Xie *et al*., [13] earlier described an efficient method for the purification of intact WSSV particles from crayfish using conventional differential centrifugations. Later Gracia-Valenzuela *et al.,* [14] reported the purification of intact WSSV virions using microfilters combined with a few steps of conventional centrifugation procedures from *Penaeus vannamei*.

This article reports a modified method for isolating intact WSSV viral particles along with high yield by combining the techniques of traditional lab centrifugation and filtration without the need for gradients or ultracentrifugation from infected *Penaeus monodon* species. In addition, the yield tracking of step-processes was studied to understand the process dynamics.

Materials and Methods

Preparation of crude WSSV inoculum

WSSV-infected *P. monodon* (15-25 g) were collected from a grow-out shrimp farm located in Gujarat, India. The presence of visible and prominent white spots on the exoskeleton served as the preliminary indication of WSSV infection which was later confirmed by WSSV PCR using vp28-F1R1 gene-specific primers that amplify a band of ~540 bp [15]. The conditions for PCR amplification included an initial denaturation at 94˚C for 5 min followed by 35 cycles at 94˚C for 20 s, 55˚C for 20s, 72˚C for 30 s, and a final extension step at 72˚C for 7 min. PCR products were analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized under UV transillumination.

The viral inoculum was prepared from, gills, pleopods, and carapace epithelium by homogenizing these tissues in a ratio of 1 g to 5 mL of TNE buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA; pH 8.0). The homogenate was centrifuged at 5000 ×g for 10 min at 4˚C, and the supernatant was filtered through a 0.45 µm membrane filter (Pall Corporation, USA). The presence of virus particles in the prepared inoculum was confirmed by PCR using vp28-F1R1 gene-specific primers as described above. The virus inoculum was stored at -70˚C until use.

Infection and proliferation of WSSV in healthy *P. monodon*

Healthy WSSV-free adult *P. monodon* (15-20 g) were acclimatized to experimental conditions for 24 h in fiberglass reinforced plastic (FRP) tanks (10 L) containing 6 L of aerated seawater (25 ppt). The prepared viral inoculum was injected (at a dilution of 1:10 in TNE buffer) intramuscularly into six healthy *P. monodon* on the ventral side of the third abdominal segment. Shrimp were observed 2-3 times daily for mortality and morbidity. The control group was injected with a blank TNE buffer. Dead and moribund shrimp were collected, monitored for WSSV infection by PCR, and kept at 4°C for virus purification.

Virus purification

5 g of infected tissues (gill, pleopods, carapace epithelium, muscle part of tail) from the infected *P. monodon* were homogenized in 25 mL TNE buffer containing 1 mM phenylmethylsulphonyl fluoride as a protease inhibitor and then centrifuged at 3000 ×g for 10 min at 4◦C. the supernatant was subjected to two more centrifugation steps at 4500 ×g and 6000 ×g. The final supernatant was first filtered through a 0.45 µm filter, followed by a 0.22 µm membrane filter (Pall Corporation, USA).

8 mL of filtered crude inoculum was then subjected to centrifugation at 35,000 ×g for 20 min at 4˚C and the supernatant was transferred to another tube. The pellet was resuspended in 4 mL of Tris-MgCl2 buffer (50mM Tris–HCl, 10mM MgCl2, pH 8) followed by centrifugation at 3500 ×g for 5 min at 4˚C. The supernatant was again centrifuged at 35,000 ×g for 30 min at 4°C sedimenting the viral particles. The resulting pellet was finally resuspended and kept in 0.5 mL Tris-MgCl2 buffer containing 0.1% Sodium azide (NaN3) and stored at -70˚C until use.

Monitoring of viral yield by qPCR

The qualitative and quantitative monitoring of all the step-process was done by PCR followed by agarose gel electrophoresis as described earlier and absolute quantification of virus copy numbers by real-time PCR using highly specific and sensitive PCR primers (vp28-140) reported earlier by Mendoza-Cano & Sánchez-Paz [16] against WSSV VP28 gene. For this, 100 µL of the virus preparation was boiled at 95˚C for 10 min followed by centrifugation at 6000 ×g. The supernatant was transferred to another tube and subsequently used as the template for qPCR using SYBR Green dye (PowerUp™ SYBR™ Green Master Mix, Applied Biosystems, USA). The reaction mixture contained 1 µL of WSSV template, 0.5 µL of each primer (10 pmol), 5 µL of 2×SYBR Green dye, and 3 µL of nuclease-free water to raise the final reaction volume to 10 µL. Real-time PCR program settings include initial denaturation at 95°C for 5 min, followed by 40 cycles of 94°C for 20 s, 61°C for 20 s, and 72°C for 20 s and a final extension step of 72°C for 5 min. The VP28 gene cloned in a plasmid was serially diluted (101–109 copies/µL) and used to generate the calibration curve for estimating virus copies in the prepared inoculum.

Integrity and viability of purified viral suspension

To observe the infectivity of isolated WSSV, an experiment was performed using healthy *P. monodon* (20-25 g body weight). The experiment was performed in duplicates with 6 adult shrimps in each replicate. Based on the previous studies, the dosage of the virus was set to 106 copies per shrimp using TNE buffer for all dilutions. Healthy shrimp injected with blank TNE buffer served as a control group. The tanks were covered with plastic sheets to prevent virus transmission by aerosol, and mortality was observed daily till 100% mortality. Shrimp were fed at 2.5% mean body weight twice a day to maintain water quality. Natural seawater was mixed with fresh water to get the desired salinity.

Results and discussion

Several protocols have been used for the isolation and purification of viable intact WSSV virions [12,13] since its discovery in penaeid shrimp in 1992[3]. These processes isolated the virions from different tissues like a gill, muscle, or hepatopancreas. All these processes have their advantages but at the same time are marred with one or another disadvantage including low yield, damaged or degraded virions, use of cost-intensive ultracentrifugation, density-gradients, and use of proteases, *etc.*

The present study reports a slightly modified method as compared to [13] by combining the techniques of gradient centrifugation and membrane filtration from WSSV-infected *P. monodon*. Additionally, the use of protease inhibitors was limited to only one to increase the viability of isolated virions. The most interesting thing about the study is the qualitative and quantitative monitoring of viral yield across all the process steps. This helps in the better understanding of the process for more improvements in further studies.

Fig-1 shows that all the process step samples that were analyzed tested positive for WSSV PCR authenticating the presence of virions in the sample. Figure 2a and 2b estimates the yield of WSSV particles in all the steps of the process. The overall yield of the pure WSSV virions was estimated as 8.64×10^{10} virions per mL (equivalent to 8.64×10^{10} virions per 5 g infected tissue). This yield is much higher than the earlier reports techniques [13, 14].

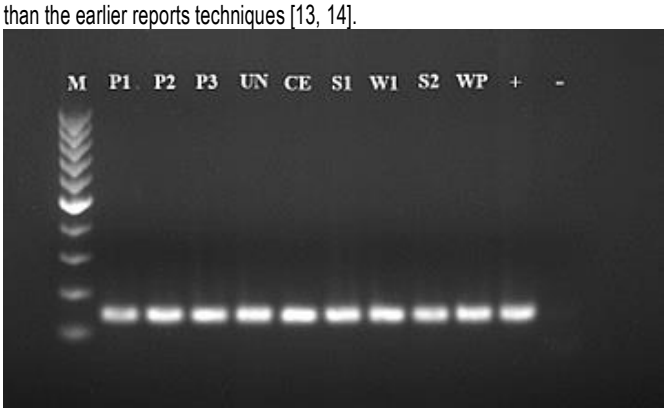

Fig-1 PCR detection of WSSV in different process steps

P1; pellet after centrifugation at 3000g, P2; pellet after centrifugation at 4500g, P3; pellet after centrifugation at 6000g, UN; Unfiltered supernatant, CE; Filtered crude extract, S1; Supernatant after first centrifugation at 35000g, W1; Pellet after 3500g centrifugation, S2; Supernatant after second centrifugation at 35000g, WP; Pure Virus suspension, +; Positive control

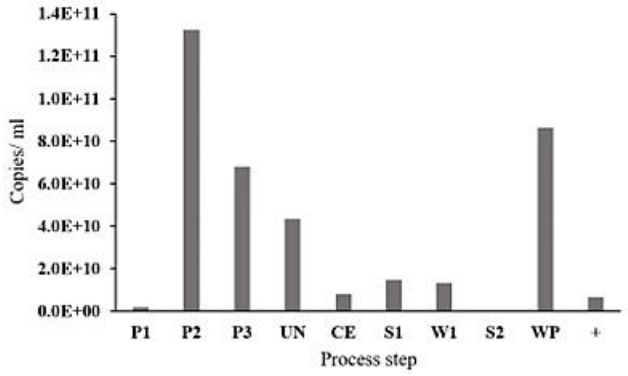

Fig-2a Complete process dynamics and yield of WSSV at different stages of purification by qPCR.

P1; pellet after centrifugation at 3000g, P2; pellet after centrifugation at 4500g, P3; pellet after centrifugation at 6000g, UN; Unfiltered supernatant, CE; Filtered crude extract, S1; Supernatant after first centrifugation at 35000g, W1; Pellet after 3500g centrifugation, S2; Supernatant after second centrifugation at 35000g, WP; Pure Virus suspension, +; Positive control

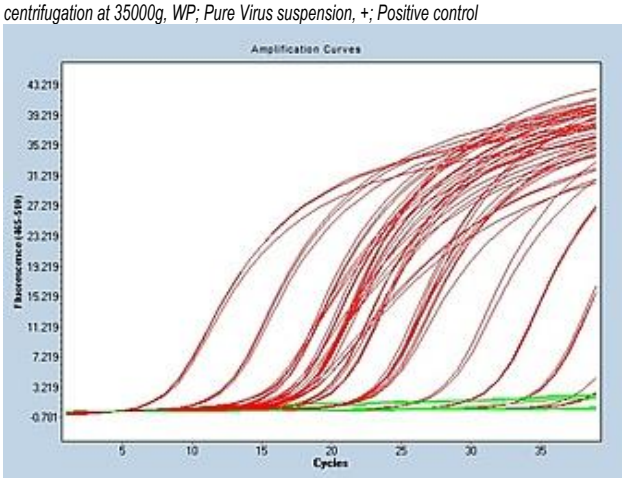

Fig-2b Real-time PCR amplification curves of standard and process samples

The integrity of the virion isolate was detected by injecting the final virus preparation (WP) into healthy shrimp. Total mortality of the adult shrimp injected with pure viral suspension was observed at 4 days post-injection. In contrast, no mortality was found in the control group injected with only TNE buffer. Clinical signs and PCR assay confirmed that the mortality was due to WSSV in 100% of the shrimp injected with WP pure viral suspension. Moreover, WSSV presence was detected in different tissues from dead organisms including pleopods, muscles, and gills.

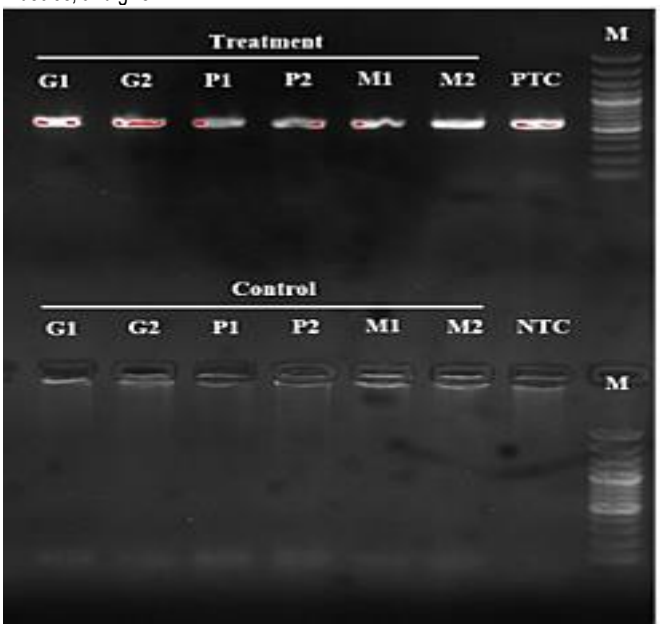

Fig-3 PCR analysis of WSSV-infected *P. monodon* DNA extracted from different tissues. G1 &G2; gill, P1 & and P2; pleopods; M1 & M2; muscle, M; Molecular marker, PTC; positive template control, NTC; negative template control

These results show that the isolated WSSV virions were viable and pathogenic. In addition, the use of a single protease inhibitor may also have increased the yield and pathogenicity of viral particles as compared to [13] who estimated the yield of the purification process at 10^{12} particles per 10 g of shrimp. However, these results cannot be regarded as conclusive as the yield of viral particles is influenced by many variables like host-pathogen interaction, stage of infection, viral strain, etc.

Conclusion

The present WSSV purification methodology and the yield-dynamic study from infected *P. monodon* will lead to more improvements in terms of simplicity and efficiency in the future. This method along with earlier reported methods for easy and inexpensive purification of WSSV virions paves way for more research concerning WSSV, its entry into the host cells, molecular pathways involved, and shrimp bioassays.

Application of research: Present research will prove helpful in conducting customized infection bioassays in shrimp and to understand the viral composition and molecular dynamics of WSSV.

Research Category: Shrimp viruses

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Research project name or number: Research station study

Author Contributions: Sole author

Author statement: Author read, reviewed, agreed and approved the final manuscript. Note-Author agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Gujarat, India

Breed name: *Penaeus monodon*

Conflict of Interest: None declared

Ethical approval/statement: The research undertaken followed the guidelines of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment & Forests (Animal Welfare Division), Govt. of India on care and use of animals in scientific research
Ethical Committee Approval Number: Nil

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