

Original Research Article

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Dynamics of Infection in Selected Tissues of White Spot Syndrome Virus-Infected *Litopenaeus vannamei*

K. Jeena^{1*}, Rahul Krishnan², K. U. Shyam², P. Gireesh Babu³,
W. S. Lakra⁴, C. S. Purushothaman⁵ and K. Pani Prasad¹

¹Aquatic Environment and Health Management Division, ICAR-Central Institute of Fisheries Education, Mumbai-61, India

²Department of Aqualife Medicine, Chonnam National University, Republic of Korea

³Fish Genetic and Biotechnology Division, ICAR-Central Institute of Fisheries Education, Mumbai-61, India

⁴ICAR-Central Institute of Fisheries Education, Mumbai-61, India

⁵ICAR-Central Marine Fisheries Research Institute, Kochi-18, India

*Corresponding author

ABSTRACT

White spot syndrome virus (WSSV) remains as the most dreaded pathogen of shrimp aquaculture since its first incidence in China in 1991. WSSV is a double stranded DNA virus belonging to the genus Whispovirus of family *Nimaviridae*. It has a wide host range and because of its high virulence it can cause 100% mortality in a period of 3-10 days. The target tissues of WSSV are of ectodermal and mesodermal origin, including gills, cuticular epithelium etc. In the present study, tissue level dynamics of white spot syndrome virus infection in *P. vannamei* was investigated. For this healthy shrimps were challenged with the virus and a time course quantification of virus load in subcuticular epithelium, gills and pleopods was carried out by Real Time PCR against the generated standard curve, which could detect as minimum as 10 copies of virus in tissue samples. In the examined tissues, the viral load increased as time progressed, however, at different degrees. Compared with gill, viral load was higher in the sub-cuticular epithelium followed by the pleopods. The study provides knowledge regarding the infectivity within the early time periods following infection. This baseline information could potentially contribute in the sensitivity determination during development of diagnostic techniques with enhanced sensitivity which can help to manage the disease to a greater extent.

Keywords

WSSV, virus load, standard curve, Real Time PCR, *L. vannamei*

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Introduction

White spot syndrome virus (WSSV) continues to be the most devastating viral pathogen infecting a wide spectrum of crustaceans and is highly pathogenic to the farmed shrimp *Penaeus vannamei*, where it is responsible for

major economic losses (Walker & Mohan 2009, Corteel *et al.*, 2012, Shi *et al.*, 2012, Yuan *et al.*, 2016). The first report on the WSSV occurrence was in China in 1991 and this was followed by other major aquaculture regions of the world including East and Southeast Asia, the Americas, India, the

Middle East, and even Europe (Verbruggen *et al.*, 2016). The total economic loss to the aquaculture industry caused by WSSV has been estimated at \$8–\$15 billion since its emergence, increasing by \$1 billion annually (Lightner, 2012; Stentiford *et al.*, 2012). The portals of WSSV entry into the crustaceans have not yet been clearly studied. Studies have shown differences in sites with respect to the entry. However, the primary sites of WSSV replication in early juvenile *Penaeus monodon* were found out as subcuticular epithelial cells of the stomach and cells in the gills, in the integument and in connective tissue of the hepatopancreas determined by *in situ* hybridization (Chang *et al.*, 1995; Di Leonardo *et al.*, 2005). The genome of WSSV has been characterized from three geographical isolates and significant studies have been made in developing various molecular diagnostic methods to detect the virus. However, the information on WSSV infection kinetics in its hosts is limited. In the present study, tissue level dynamics of white spot syndrome virus infection in *P. vannamei* was investigated. This baseline study will be helpful in predicting the sensitivity of different diagnostic tests and in finding the minimal infection level at which the disease could be managed.

Materials and Methods

Experimental animals

American white shrimp, *Penaeus vannamei* were procured from D'souza farm, Naigaon, Maharashtra. Live and healthy shrimps weighing 15 ± 2 g were transported in plastic containers with proper aeration and were given dip treatment in 2 ppm potassium permanganate to avoid any infection due to injury and stress. Shrimps were screened for the absence of all the viruses using PCR and were maintained at 28 °C and salinity of 20 g/L with continuous aeration in wet lab of

ICAR-CIFE. Animals were acclimated for 7 d prior to experimental treatments and fed *ad-libitum* 2 times a day with pelleted feed.

Viral inoculum preparation and WSSV challenge

White spot syndrome virus inocula were prepared by homogenizing the infected shrimp muscle tissue in phosphate-buffered saline (PBS). The homogenate was centrifuged at 8000 g for 10 min and the supernatant collected was filtered using a 0.45 µm PVDF syringe filter. This supernatant was used to inoculate the experimental shrimp. Prior to the injection, the viral load was determined using real-time PCR. *In vivo* titration revealed that the injection of 5×10^6 copies of virion particles into shrimp resulted in 100% mortality within 5 days post infection. Animals were injected with the viral inoculum (5×10^6 copies) 100 µl, ventral to the second abdominal segment and were housed individually in tanks.

Tissue collection

Tissues from three infected shrimps were collected at each time point for the assays. Gill, sub-cuticular epithelium and pleopod were collected from each shrimp at 0, 1, 3, 6, 12, 24, 48 and 72 h post infection (hpi) and processed for nucleic acid isolation

Isolation of nucleic acids

Tissues from three animals belonging to the same time point were pooled and DNA was extracted separately from gills, sub-cuticular epithelium and pleopod using the method of Sambrook *et al.*, (2001) with some modifications. The DNA concentration and quality were estimated using a nano volume spectrophotometer and the concentration of DNA were adjusted to 100 ng/µL using nuclease free water.

Construction of positive control vectors and standards for quantification

The WSSV-VP28 qPCR primers developed by Mendoza-Cano and Sánchez-Paz (2013) were used in the present study. WSSV VP28 fragment containing a 171 bp target amplicon was ligated into the pTZ57R/T vector and transformed into *E.coli* DH5 α .

The plasmid DNA were purified using Miniprep plasmid purification kit (ThermoFisher Scientific, USA) and the plasmid was sent for sequencing at Bioserve Biotechnologies India Pvt. Ltd., (Hyderabad, India) for further confirmation of the clones. The copy number of the target amplicon in the plasmid was estimated and tenfold serial dilution was used as absolute standards for quantification using real-time PCR.

Real-time PCR amplification

To determine the WSSV load in different tissues at various time points, a SYBR Green based qPCR assay was carried out in LC96 Light cycler (Roche, Germany). The amplifications were performed in a 96-well format with 10 μ L final volume containing 5 μ L of SYBR Green master mix (Takara, Japan), 0.2 μ L of (10 pM) each forward and reverse primers, 1 μ L each of DNA dilutions as template.

The reaction was performed in triplicates along with non-template controls to rule out the cross contamination. The thermal profile was 95°C for 30 s, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s and extension at 72°C for 10 s. A series of dilutions of VP28 plasmid were used for generating a standard curve. Analysis of variance (ANOVA) test was used to compare the mean viral copy number at different time points. A Tukey post-hoc test was used to determine significant difference in

the viral copy number among tissues at a particular time. All statistical tests were performed using the program SPSS 22.0 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Standard curve

The optimized PCR conditions suggested in the previous studies were applied to develop a standard curve using tenfold serial dilutions of the plasmid DNA. A representative amplification plot generated for the plasmid standard (ranging from 1×10^{10} to 1×10^1 copies per reaction) is shown in Fig. 1; each dilution for this experiment was performed in triplicate. The melting curve analysis revealed a single peak for the amplified product with a melting temperature (T_m) of 84.8°C (data not shown). A linear correlation ($R^2 = 0.955$) was obtained between threshold cycles (CTs), indicating a detection limit of 10 copies. The slope of $M = 3.39$ is indicative of high reproducibility and precision (Fig. 2).

Viral load in WSSV-infected tissues

White spot syndrome virus DNA was detected in all the *P. vannamei* tissues by real-time PCR. The tissue level viral load as copy numbers was determined against the generated standard curve (Fig. 2). In all tissues, the viral load increased as time progressed, however, at different degrees. (Fig. 3).

Compared with gill, viral load was higher in the sub-cuticular epithelium followed by the pleopods. In the gill, 2.76×10^1 WSSV DNA copies/ μ g of total DNA were detected at 6 hpi and levels continued to increase to 1.85×10^2 , 1.5×10^4 and 3.81×10^6 at 12, 48 and 72 hpi. Compared with gills, WSSV DNA levels in sub-cuticular epithelium showed a tenfold increase at the first sampling (2.97×10^2 WSSV DNA copies μ g/L of total DNA at 12 hpi).

Fig.1 Amplification curve showing tenfold serial dilutions of standard samples. Representative plot is depicted. The experiment was run in triplicates

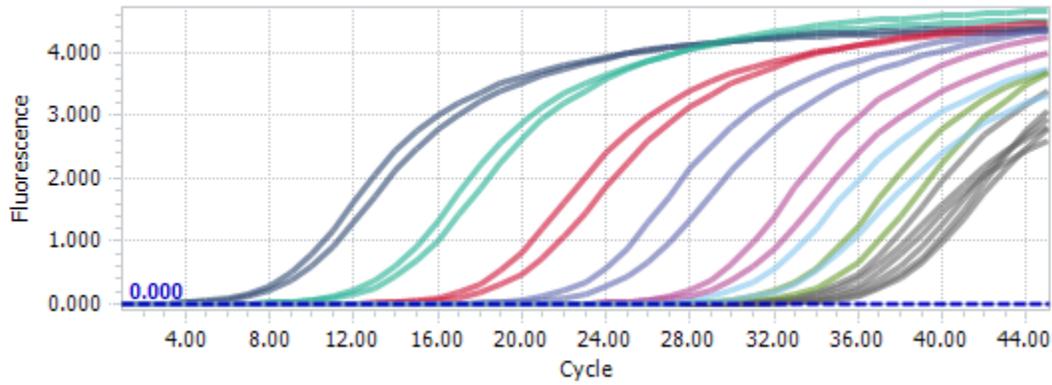


Fig.2 Standard curve of WSSV real-time PCR; C_q, threshold cycles. The correlation coefficient was 0.955, and slope of the line was 3.39

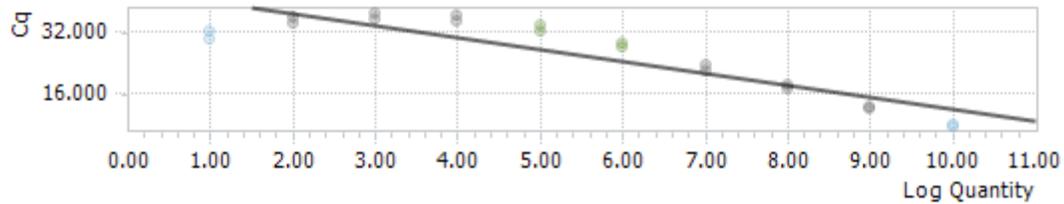
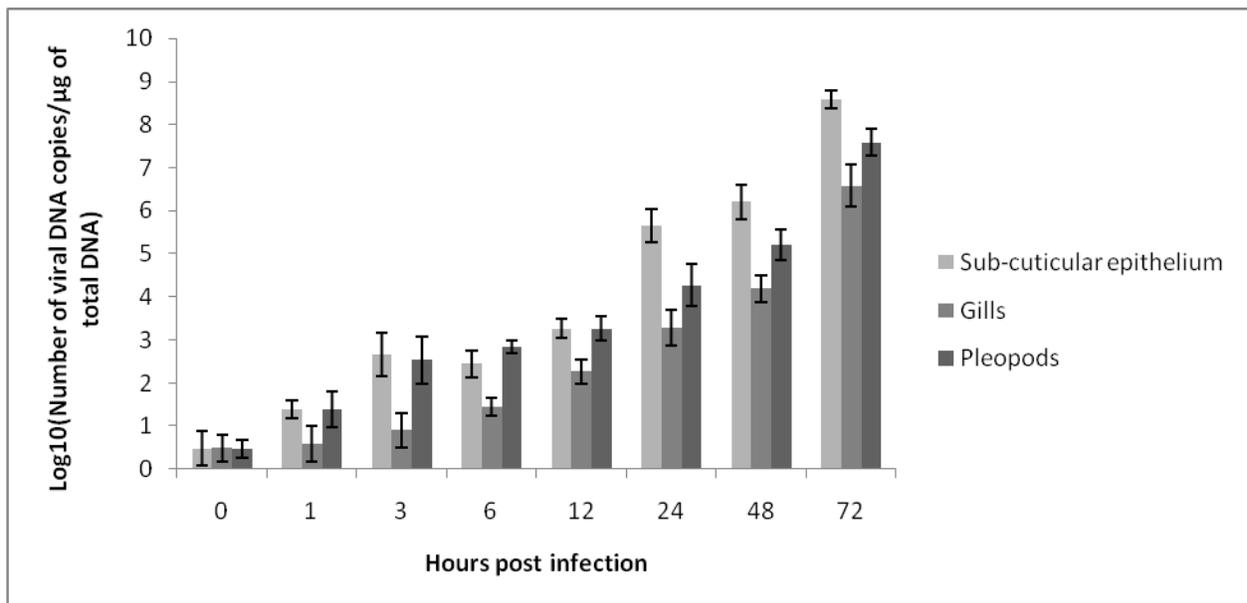


Fig.3 Tissue distribution of WSSV following challenge at different time points. Data represented as mean value of triplicate experiments with standard error. All the values are statistically significant ($P < 0.05$)



There was no significant difference in the WSSV load between the pleopod and sub-cuticular epithelium till 12 hpi, later it was about tenfold lower at 24 hpi (1.8×10^4), and about 100-fold lower at 48 and 72 hpi (1.5×10^5 , 3.8×10^7). The viral copy number of tissues at different time intervals, analyzed using one-way analysis of variance (ANOVA), showed a significant difference between tissues at all-time points ($P < 0.001$). Subsequent pair-wise analysis of WSSV copy number in different tissues at a particular time point.

The present study determined the time-dependent viral load in defined tissues of *P. vannamei* experimentally infected with WSSV. A sensitive SYBR green real-time PCR assay developed by Mendoza-Cano and Sánchez-Paz (2013) was adopted. Till date, studies on tissue distribution of WSSV in infected penaeid shrimps are scarce. Durand and Lightner (2002) evaluated the WSSV load in various tissues of different shrimp species using moribund juveniles. Moribund juveniles of *P. vannamei* showed 2.5×10^9 , 1.6×10^9 , 1.2×10^9 and 1.9×10^8 copies of WSSV from haemolymph, pleopod, gills and muscle respectively. Moribund juveniles of *P. stylirostris* and *P. monodon* showed 3×10^{10} and 2.1×10^6 WSSV copies in pleopod. This implies that large variations in viral load can be observed between different tissues in the same species and between the same tissues in different shrimp species. A similar trend was observed in the present study showing higher load in the sub cuticular epithelium followed by the pleopod and the gill.

This study demonstrates the viral replication kinetics in each of the tissues during the course of viral replication over a period of time. At 6 hpi, sub-cuticular epithelium and pleopod showed relative high copy number followed by gills (Fig.3). Over time, a rapid increase in viral load is observed in sub-

cuticular epithelium. At 24, 48 and 72 hpi, sub-cuticular epithelium showed the highest viral load among all tissues analyzed. According to Escobedo-Bonilla, *et al.*, (2007), the mechanism behind such change in viral distribution is that after primary replication (12 or 18 hpi), newly produced WSSV would have been released from epithelial cells and crossed the basal membrane to reach the underlying connective tissues.

On the contrary studies of Jeswin *et al.*, (2015) demonstrated that highest viral load followed by WSSV experimental infection was higher in haemocytes at the initial stages and further advanced with a higher copy number in the gills at 24, 36 and 72 hpi in *P. monodon*. WSSV can replicate in all the vital organs of infected penaeid shrimps. The target tissues for WSSV replication includes the epidermis, foregut, gills, antennal gland, hindgut, gonads, lymphoid organ, hematopoietic cells, cells associated with the nervous system, and connective tissue whereas tissues, like hepatopancreas and gut, are refractory to WSSV infection (Zhao *et al.*, 2017).

This study demonstrates the time-dependent WSSV infection kinetics in different tissues and provides knowledge regarding the infectivity within the early time periods following infection. This baseline information could potentially contribute in the sensitivity determination during a diagnostic development and also help to manage the disease to a greater extent.

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