Detection of white spot syndrome virus (WSSV) in the pacific white shrimp *Litopenaeus vannamei* in southern India using PCR, SEM and histological techniques

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Outbreak of disease is one of the major stumbling blocks in the development and sustainability of aquaculture. White spot syndrome virus (WSSV), the causative virus of the white spot disease, was found in most shrimp farming areas of the world, where it causes large economic losses to the shrimp farming industry. The objective of the present investigation was to determine the level of WSSV transmission from the infected tiger prawn *Penaeus monodon* to specific pathogen free (SPF) *Litopenaeus vannamei* in WSSV impact region of Tamil Nadu. In our study, we performed polymerase chain reaction (PCR), histological observation and scanning electron microscopy to detect WSSV. Our nested PCR revealed the bands ranging from 296 to 910 bps in WSSV infected *L. vannamei* shrimps. The infected shrimp showed 20 to 2000 copies of viral DNA. Histological surveillance was performed on infected *L. vannamei* at cephalothorax region. We observed intranuclear inclusion bodies characteristic in the gill region of infected *L.vannamei*. No significant difference was noticed in the hematopoietic tubules of WSSV affected *L.vannamei*. However, in the higher magnification, more than 15 kV × 150 the hematopoietic tubules were found at 10 µm in diameter. Basically, the study was initiated to document the primary instance of WSSV infected shrimp, which was new imported species. Overall, the investigation provides information on the level of pathogenicity of WSSV in different species of *L. vannamei* including SPF and native species.

**Keywords:** *Litopenaeus vannamei*, PCR, *Penaeus monodon*, SEM, white spot syndrome virus (WSSV)

**Introduction**

White spot syndrome virus (WSSV) is one of the most serious pathogens affecting the shrimp industry worldwide. WSSV is the sole member of the novel genus *Whispovirus* within the family *Nimaviridae*. WSSV is a large (80-120×250-380 nm), rod to elliptica-shaped virus with a trilaminar envelope and double-stranded DNA (dsDNA). The WSSV disease is an economically significant shrimp disease, which causes high mortalities and severe damages to shrimp cultures. The principle clinical sign of the affected shrimp is white spots on the exoskeleton and epidermis. This virus can cause 100% mortalities within 3 to 10 d on the onset of symptoms. WSSV has a wide host range and infects shrimps, crabs and arthropods, such as, copepods insects and pest prawns.

The first major WSSV outbreak was reported in 1993, which resulted in a 70% reduction in shrimp production in China. Since then it remained a major concern for shrimp aquaculture throughout the world. The presence of WSSV has been reported in both wild and hatchery reared post larvae. WSSV has become an epizootic disease and is not only a major threat to shrimp aquaculture, but also to marine ecology. The development of robust detection methods and studies on the ecology of WSSV in wild populations should be treated as priority. Otherwise, a complete evaluation of the potential risk as a result of direct or indirect exposure of wild organisms to this virus will be impractical. In many cases, post-mortem necropsy and histology have been the primary methods for the diagnosis of fish and shellfish diseases. However, these methods often lack specificity and many pathogens are difficult to detect when present in low numbers or when there are no clinical signs of diseases.

In the present study, we determined the transmission level of WSSV from the infected tiger prawn, *Penaeus monodon* to specific pathogen free (SPF) *Litopenaeus vannamei* in the affected region of...
Tamil Nadu. The WSSV infected shrimps were examined for the presence of virus in lymphoid organs, hematopoietic tubules and hepatopancreatic tissues, gills and hind gut region. The presence of WSSV in different tissues was also studied by SEM, PCR and tissue histology.

Materials and Methods

Study Area and Sampling Methods

A large numbers of aquaculture farms are in operation in the Southeast coast of India (Tamil Nadu). Twelve study sites were chosen from Pulicate to Tuticorin (Fig. 1) and 600 number of *L. vannamei* shrimp samples from these sites were collected at an average of 50 shrimp in each locality. Adult shrimp weighing 10-15 g were randomly collected from different aquaculture environments of the study site. The sampling was done prior to the time of harvest in the hatcheries. In fact, these shrimps were imported from aquaculture farms of Brazil to the Indian hatcheries through different private sectors. Collected samples were stored at −20°C for further use. DNA was isolated from gills, appendages, and pleopod legs from adult shrimps and larvae.

Sample Preservation

The tissue samples for DNA extraction was cut into small pieces (< 5-7 mm) to permit adequate fluid penetration and preserved in fresh 95% ethanol using 1.5 mL labeled tubes. The ethanol was usually poured off after few days of collection and replaced with fresh 95% ethanol to optimize DNA preservation. The tubes were stored under refrigerated condition.

DNA Extraction

DNA was extracted from each sample using the DNA extraction kit IQ2000 WSSV (Farming IntelliGene Tech. Corp., Taiwan). The tissues dissected from pleopods and gills were replaced in 2 mL Eppendorf tubes containing 0.6 mL of a premixed dodecyl-trimethylammonium bromide (DTAB) solution and emulsified with disposable grinding pestles. The samples were incubated in a heating block at 75°C for 5 min and then cooled at room temperature. The mixture was then mixed briefly and centrifuged 12,000 rpm for 5 min. About 0.7 mL of chloroform was added and then briefly vortexed, followed by centrifugation at 12,000 rpm for 5 min. The upper aqueous phase was transferred to a new 2 mL Eppendorf tube, mixed briefly with 100 µL of a premixed N-cetyl-N,N trimethylammonium bromide (CTAB) solution and 900 µL double-distilled-water, and incubated at 75°C for 5 min before cooling at room temperature. After centrifugation at 12,000 rpm for 5 min, supernatant was transferred and the pellet was resuspended with 150 µL of dissolving solution (provided with the kit). After incubation at 75°C for 5 min the samples were again centrifuged at 12,000 rpm for 5 min and the supernatant was transferred to a fresh 0.5 mL eppendorf tube mixed with 300 µL 95% ethanol, vortexed and centrifuged at 12,000 rpm for 5 min. The pellet was then washed with 200 µL 70% ethanol, dried at room temperature and resuspended in 100 µL of TE buffer (10mM Tris, 1mM EDTA, under pH 8.0).

Virus Purification

Viral DNA was isolated from purified virions by treatment with proteinase K (0.2 mg/mL) and Sarkosyl (1%) at 65°C for 2 h, followed by phenol and chloroform extraction and dialysis against TE buffer. The extracted DNA was determined by 1.5% agarose gel electrophoresis.

PCR Analysis for the level of WSSV infection

WSSV-DNA was detected from each sample using a commercial 2-step PCR detection kit (OIE recommended 9195/9992 primers). The PCR was performed using the method of 2-step nested PCR, sequential semi-quantitative assay targeting a conserved region of WSSV-DNA sequence, described by IQ2000 Farming IntelliGene Tech. Corp, Taipei, Taiwan. In the first step, a 910 bps fragment was amplified. 2 mL of DNA template was added to 8 mL PCR Pre-Mix reagent and 0.5 mL of IQzyme DNA polymerase.
Paraffin oil was used to cover the mixture and PCR was conducted as follows: 5 cycles at 94°C for 20 sec, 62°C for 20 sec and 72°C for 20 sec; 15 cycles at 94°C for 10 sec, 62°C for 10 sec and 72°C for 15 sec; and a final extension cycle at 72°C for 30 sec. In the second step, PCR Pre-Mix reagent including nested primers and IQzyme DNA polymerase (2 U/mL) were added to amplify three fragments (910, 550, 296 bps) from the initial amplification product. PCR was conducted as follows: 25 cycles of 94°C for 20 sec, 62°C for 20 sec, 72°C for 30 sec, followed by 72°C for 30 sec. A series of dilutions of WSSV plasmid DNA containing $10^3$, $10^2$ and $10^1$ copies (supplied with the kit) were used as positive standards, and yeast tRNA (also supplied with the kit) was used as a negative control.

After amplification, 10 µL of the PCR product was mixed with 2 µL of 6× loading dye (provided with the kit) and 5 µL DNA mol markers loaded onto a 1.5% agarose gel with 1× TBE (Trizma, boric acid, EDTA) buffer, containing 0.5 µg/mL ethidium bromide. Three serial dilutions $10^1$, $10^2$ and $10^3$ (supplied with the kit) of WSSV plasmid DNA were used as positive controls to interpret as light, medium or severe infections, respectively. Electrophoresis was performed in 1× TAE buffer (20 mM Tris-HCl pH 7.5, 20 mM glacial acetic acid, 0.5 mM EDTA, pH 8.0) at 100 V for ~30 min. Amplified fragments were visualised under ultraviolet transillumination exposed GelDoc System and photographed.

Histology

The WSSV infected and non-infected organs from shrimps were examined by histology for WSSV-specific manifestations following the routine diagnostic protocol of Lightner. The Davidson’s fixative (needle gauge was selected based upon the shrimp sizes) was injected into the live shrimp in the mid-gut gland, posterior abdominal region and in the anterior abdominal region. The procedure was followed under the instruction of OIE standard text Manual of Diagnostics Tests for Aquatic Animals 2009.

SEM-Scanning Electron Microscope

Tissues were dissected and then cut into smaller pieces in sizes ranging from 2 mm³. The primary fixation of tissues was done for 1-4 h at 4°C in 2.5% glutaraldehyde in 0.1 M buffer, pH 7.4. The samples were then washed for 2 h at 4°C for 3 repeated changes of 0.1 M buffer, pH 7.4. The fixative should be 10 times greater in volume comparing to the specimen and the tissue samples were maintained in wet condition. The Secondary fixation was done for 1 h at room temperature in 1% osmium tetroxide in distilled water and washed for 2-5 min with distilled water. After dehydration, the samples were treated with propylene oxide and resin mixture in 1:1 ratio for 2-10 min. The samples were again treated with fresh resin and placed in a rotary mixer at 5 rpm overnight. Then they were embedded in mould and polymerized in oven at 60°C for 24 h. The samples were then analyzed by SEM.

Results

Prevalence of WSSV

The distribution analysis from 12 study sites showed that 7 sites were having normal healthy shrimp and 5 sites were having WSSV infection. The prevalence percentage frequencies of infection observed in these sites are represented in Table 1.

<table>
<thead>
<tr>
<th>No.</th>
<th>Endemic site</th>
<th>No. of sample collected</th>
<th>WSSV</th>
<th>WSSV copies</th>
<th>Mol base pairs (bps)</th>
<th>Infection level</th>
<th>Prevalence (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Pulicat</td>
<td>50</td>
<td>Positive</td>
<td>20</td>
<td>296</td>
<td>Low</td>
<td>100</td>
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<tr>
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<td>Kallapakkam</td>
<td>50</td>
<td>Positive</td>
<td>200</td>
<td>550, 296</td>
<td>Severe</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Marakkam</td>
<td>50</td>
<td>Positive</td>
<td>200</td>
<td>550, 296</td>
<td>Severe</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>Parangipettai</td>
<td>50</td>
<td>Positive</td>
<td>2000</td>
<td>910,550,296</td>
<td>Severe</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>Sirkazhi</td>
<td>50</td>
<td>Positive</td>
<td>2000</td>
<td>910,550,296</td>
<td>Severe</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>Karaikal</td>
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<td>848</td>
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<td>0</td>
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<tr>
<td>7</td>
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<td>Nil</td>
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<tr>
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<td>Nil</td>
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</table>
Detection of WSSV by Nested PCR

The healthy normal uninfected and WSSV infected *L. vannamei* shrimp are shown in Figs 2a and b. From our study area (*L. vannamei* shrimp), we randomly selected shrimps from both normal and WSSV infected (low and severe WSSV infection) condition and analysed the viral genes by nested PCR using gene specific primers for the infection. As expected, we detected the expected size of amplifications for WSSV from the infected samples (gills of infected shrimp) and not in healthy shrimps (Figs 3 & 4).

Our nested PCR detected different sizes of expected viral specific bands, clearly indicating the severity of infection that also correlates with the copy numbers of particular gene(s) amplification. For example, in low infection condition, we detected 296 bp fragment with nearly 20 copies (Fig. 4; lane 2; sample 1) of amplification and 550 and 910 bp in moderate and severe infection with more than 200 and 2000 copies (Fig. 4; lanes 8, 12, 13 & 15; samples 2-5), respectively. In all the experiments, internal control primers, which amplify only host specific genes, were included. WSSV negative samples were indicated at 848 bp in non-infected sites (Fig 3; lanes 4-7; samples 6-9 & Fig. 4; lanes 5, 6 & 11; samples 10-12).

Histological Observation of WSSV

WSSV infected *L. vannamei* shrimps were collected from selected study area (Fig. 1) and different tissues and organs were dissected out for histopathological observations using standard H and E staining. The basophilic intranuclear inclusion bodies were seen in the hematopoietic tissues of WSSV infected *L. vannamei* (Fig. 5). Very clear intranuclear...
inclusion bodies were also observed in other tissues, such as, hepatopancreas, gill and hind gut region of WSSV infected *L. vannamei* (Figs 6-8).

**Scanning Electron Microscopic Observation of WSSV Infection in *L. vannamei***

In order to check whether there was any change in appearance or deformities of important tissues or organs of *L. vannamei* due to WSSV infection, we collected different tissues and organs including gut, hepatopancreas, gills, central nervous system, lymphoid organ and cardiac muscle from WSSV infected *L. vannamei* and observed under scanning electron microscope. Fig. 9 shows the granules like whitish spots in the size of 20 µm in diam on the carapace of WSSV infected shrimps. A blister like appearance and partial damage were seen in the lymphoid organ of WSSV infected *L. vannamei* (Fig. 10). The same blister appearance was also observed in hepatopancreas tissues (Fig. 11). Observation from WSSV infected shrimp further

![Fig 6—Intranuclear inclusion bodies in hepatopancreas of WSSV infected *L. vannamei*.](image)

![Fig 7—Intranuclear inclusion bodies in gill lamella of WSSV infected *L. vannamei*.](image)

![Fig 8—Intranuclear inclusion bodies in hind gut of WSSV infected *L. vannamei*.](image)

![Fig 9—Granules like whitish spots on the carapace of WSSV infected *L. vannamei*.](image)

![Fig 10—Blister like appearance and partial damage on the lymphoid organ of WSSV infected *L. vannamei*.](image)

![Fig 11—Blistering appearances of hepatopancreas tissue of WSSV infected *L. vannamei*.](image)
showed clear dense white spots appearance in central nervous system (Fig. 12). A small numbers of tiny spots were observed in cardiac muscle tissues of WSSV infected shrimp (Fig. 13). Noticeable patches like appearance were also seen in gill lamella and hematopoietic tubules (Figs 14 & 15).

**Discussion**

In the present study, we selected different areas in Tamil Nadu, India, which cultivate *L. vannamei* shrimp and investigated the WSSV infection by PCR and its effect on host internal organs using histological and scanning electron microscopy. Our nested PCR from different study areas identified low to severe infection for WSSV infection with its respective PCR amplifications (Fig. 4). Among the study sites, Kalpakkam, Marakkanam, Parangipettai and Sirkali, which are located between Pulicat to Tuticorin, were shown to be affected severely by WSSV and the rest of the study sites were shown negative results for WSSV infection. The infection could be due to the location of shrimp farms near the vicinity of infected ponds or the adjacent farms cultivating native species *P. monodon*, which is more susceptible to WSSV.

Comparable differences in susceptibility to viral, bacterial and fungal infection among penaeid shrimps have been reported earlier. In the present study, using histopathological and SEM examinations, we observed several changes or appearances of several vital organs of WSSV infected shrimp *L. vannamei* species. Importantly, the lichen like granules on the top of the exoskeleton revealed the presence of WSSV on the carapace (Fig. 9) of infected shrimp. However, the occurrence of whitish spots was intensively noticed on the lateral region of the carapace, which were seen with the size of 20 µm and 10 µm in diam. The white spots were noticed with the size of 5 µm in the epidermal layer of the carapace of *L. vannamei* in the higher magnification under SEM. At higher magnification, the infected lymphoid organ, dorso-lateral area, appeared as spheroids with cavity containing cluster of WSSV. The spot was detected
by the presence of radiating lines with 10 µm in diam on the lateral region of the lymphoid organs as seen in the lower magnification in SEM with the size of 10 µm (Fig. 10). Spheroids are a typical form of WSSV infected lymphoid organ, which was observed here in WSSV infected L. vannamei.

In addition to PCR detection for viral infection, our results clearly showed the destruction of several important host organs and tissues including hepatopancreatic tubules, central nervous system and cardiac muscle tissues in WSSV infected L. vannamei, suggesting that WSSV destroy the host tissues completely in order to maintain its own survival. Overall, this study provides information on how WSSV infection affects the host internal tissues and organs with different appearances, which would help in developing new identification methods for early detection and new safety measures against WSSV infection.

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References