Protein Profiles Of Pacific White Shrimp (*Litopenaeus Vannamei*) And Hermit Crab, (*Clibanarius Longitarsus*) Before And After WSSV Infection

B. Visnu[1*], S. Priyangha[1], S.T. Somasundaram[1], S. Bragadeeswaran[1], S. Kumaresan[1], R. Seetharaman[1], T. Sasidharan[1], S. Srikavibarathi[1], J.Mohamed Asarudeen[1]

[1*]Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608502, Cuddalore District, Tamil Nadu, India

***Corresponding author:** B Visnu
*Research Scholar, Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608502, Cuddalore District, Tamil Nadu, India, Email: visnubala1997@outlook.com

Abstract
The prevalence of White Spot Syndrome Virus (WSSV) infection in the cultured shrimps is a havoc and leads to devastation of the aquaculture industry. In this backdrop, the present study is designed to investigate the changes in protein profile before and after infection for the candidate species *Litopenaeus vannamei*. The hermit crab *Clibanarius longitarsus* was also chosen for this investigation as it is known for sturdy against viral infection. Hence, it is determined to undergo protein profile study in order to light up the path of drug design in the near future. Both the candidate species were confirmed for non-infectious using DNA based molecular diagnostic (PCR) method and subsequently the experiment was initiated in the laboratory by making infection with WSSV through feeding with WSSV-positive tissues. The infection was confirmed in the course of challenge test by collecting the hemolymph, isolating the DNA and amplifying using PCR, and running on gel electrophoresis. For further confirmation, SDS PAGE method has been performed for the healthy and WSSV infected *Litopenaeus vannamei* and *Clibanarius longitarsus.* The gel electrophoresis of SDS PAGE reveals the expression of Heat Shock Protein (HSP 60) which is a signal cascade that is expressed as an immune response. This investigation on the proteomics showed promising results between the species of highly vulnerable *Litopenaeus vannamei* and asymptomatic *Clibanarius longitarsus*.

Keywords: Aquaculture, Diseases, WSSV, Diseases Resistance, Protein Profiling

INTRODUCTION

Currently, more than 220 aquatic species are cultured, with the total worldwide production amounting to over 90 million tonnes. Ten of the top 14 aquaculture-producing countries are located in Asia. This global amount compares with just over 92 million tons of aquatic products obtained from harvesting wild species. The prediction is that aquaculture will continue to grow (the current annual growth rate is around 7%), quickly overtake the harvesting of wild species, and become the dominant provider of aquatic plants and animals, worldwide.

Huge economic losses occur due to the incidence of viral and other diseases in shrimp farms in India. The gross national losses in the country due to shrimp diseases was estimated at 48717 metric tons of shrimp worth of above Rs. 1022.1 crores, and employment of 2.15 million man-days.

The introduction of *Litopenaeus vannamei* in Asia increased shrimp production significantly. Among different species, *L. vannamei* production stands 70% and the rest includes *Penaeus monodon, Macrobrachium rossenbergii, P. chinensis, P. merguiensis, P. japonicas,* and *P. indicus* etc. The increase in demand for shrimps has resulted in an intensification of production, which is directly linked with an increased incidence of diseases. Diseases, in particular, are a major constraint to the sustainability of shrimp production in many countries (Supamattaya *et al*.,1998)

WHITE SPOT SYNDROME VIRUS (WSSV)

It is an enveloped virus with a 300-kb circular double-strand DNA that infects a wide range of decapod and non-decapod crustacean hosts in natural environments. Clinical signs of WSSV include a sudden reduction in food consumption, lethargy, loose cuticle, and often reddish discoloration, and the presence of white spots of 0.5 to 2.0 mm in diameter on the surface of the carapace, appendages, and cuticle over the abdominal segments. In the host, WSSV infects a wide variety of cells of ectodermal and mesodermal origin. Histological changes are seen in the gill epithelium, antennal gland, hematopoietic tissue, nervous tissue, connective tissue, and intestinal epithelial tissue. Infected cells have prominent intranuclear occlusions that initially stain eosinophilic, but become basophilic with age; hypertrophied nuclei with chromatin margination; and cytoplasmic clearing. (Wongteerasupaya, *et al*., 1995)

White spots on the shell of infected shrimp under scanning electron microscope appear as large, dome-shaped spots on the carapace measuring 0.3 to 3.0 mm in diameter. Smaller white spots of 0.02 to 0.10 mm appear as linked spheres on the cuticle surface. The chemical composition of the spots is similar to the carapace, calcium forming 80–90% of the total material, and may have derived from abnormalities of the cuticular epidermis. (Wang, *et al*., 2018) several biochemical changes have been reported after infection with this virus glucose consumption and plasma lactate concentration increase, glucose 6 phosphate dehydrogenase activity increases, and triglyceride concentration decreases. The voltage-dependent anion channel of the mitochondrion is also unregulated.

MATERIALS AND METHODS SAMPLE COLLECTION AND GROSS OBSERVATION
The collected samples were observed for WSSV and its gross sign of symptoms of infection.

WET MOUNT OBSERVATION OF INFECTED SPECIMENS
The carapace of the infected specimens was washed with distilled water sectioned using a scissor and it was mounted on a glass slide with water, and viewed under the light microscope at various magnifications to check WSSV symptoms and its positivity.

PCR AMPLIFICATION
WSSV-infection was confirmed by the PCR technique using the primers of (Yoganandhan *et al*., 2003), RT-PCR at the end of the experiment. Template DNA for PCR tests was prepared from experimental animals by extraction from gills, following the method described by (Lo *et al*., 1996). Gill tissues were cut and homogenized. The homogenized gill tissue sample was centrifuged at 3000× g at 4 °C, after which, the supernatant fluid was placed in another centrifuge tube together with an appropriate amount of digestion buffer (100 mM NaCl, 10 mM Tris HCl, 50 mM EDTA, 0.5% sodium dodecyl sulfate, 0.1 mg/ml proteinase K, pH 8.0). After incubation at 65 °C for 2 hrs, the digest was deproteinized by successive phenol/chloroform/isoamyl alcohol extraction, recovered by ethanol precipitation and dried; the dried DNA pellet was resuspended in TE buffer. For PCR, the primers designed by (Yoganandhan *et al*. 2003) were used to amplify a 200-bp fragment of WSSV-DNA. PCR products were analysed by electrophoresis in 1% agarose gels stained with ethidium bromide, and visualized by ultraviolet transillumination.

PCR AMPLIFICATION
WSSV-infection was confirmed by the PCR technique using the primers of (Yoganandhan, *et al*., 2003), bioassay, RT-PCR and Western blot analysis (Sahul Hameed *et al*., 1998) at the end of the experiment. Template DNA for PCR tests was prepared from experimental animals by extraction from gills, following the method described by (Lo, *et al*., 1996). Gill tissues were cut and homogenized. The homogenized gill tissue sample was centrifuged at 3000× g at 4 °C, after which, the supernatant fluid was placed in another centrifuge tube together with an appropriate amount of digestion buffer (100 mM NaCl, 10 mM Tris HCl, 50 mM EDTA, 0.5% sodium dodecyl sulfate, 0.1 mg/ml proteinase K, pH 8.0). After incubation at 65 °C for 2 h, the digest was deproteinized by successive phenol/chloroform/isoamyl alcohol extraction, recovered by ethanol precipitation and dried; the dried DNA pellet was resuspended in TE buffer. For PCR, the primers designed by (Yoganandhan, *et al*., 2003) were used to amplify a 200-bp fragment of WSSV-DNA. PCR products were analysed by electrophoresis in 1% agarose gels stained with ethidium bromide, and visualized by ultraviolet transillumination.

Procedure of Protein Extraction
Fresh tissue samples were finely ground with mortar and pestal. Crushed materials (0.02g) were transferred to each 1.5 ml Eppendorf tube with addition of 400 µl protein extraction buffers (0.5M Tris-HCl (pH 8.0), 0.2% Sodium dodecyle sulphate (SDS), 5M urea, 1% 2-mercaptoethanol, and bromophenol blue dye). The samples were properly mixed by vertexes for 1-2 minutes and stored overnight in refrigerator at -20℃

SDS PAGE
Preparation of separation and stacking gels the separation and staking gels were prepared by mixing chemicals in 10% concentrations. The samples were then centrifuged at 12000 rpm for 10 minutes. 10 µL upper layer of each sample was loaded to each well along with protein marker at 100 V.

The moment of proteins was noted regularly until reach at the bottom of plates. The gels were then transferred into staining solution and kept for 2-3 hours on shaker. The gels were then washed two times with distilled water and then transferred into fresh de-staining solution and kept on shaker for 24 hours.

The autoclave tissue paper was also kept on gel to remove excess of blue colour. The bands patterns of all genotypes were noted. (Sneath and Sokal, 1973).

RESULTS SAMPLE COLLECTION WSSV infected animals

White spot syndrome virus infected *Penaeus monodon* was collected from Vellar estuary (Fig 1). The samples of WSSV infected were collected by confirming white spots on the carapace. The animals were brought to the laboratory in ice-cold conditions and further processed to confirm the prevalence of infection.

Fig 1: *Penaeus monodon* with WSSV

Experimental Animal

Healthy *Litopenaeus vannamei* was collected from a commercial shrimp farm that has no earlier history of WSSV and healthy *Clibanarius longitarsus* was also collected from the Vellar estuary to perform the experiment (Fig 2). The collected animals were maintained in quarantine tanks for a week and then the infectivity study has been started.

Fig 2: Study animals *Clibanarius longitarsus* and *Litopenaeus vannamei*

Identification

The shrimp and crab samples collected were morphologically identified based on FAO as *Penaeus monodon*,(Fabricius 1798), *Litopenaeus vannamei,* and *Clibanarius longitarsus*

Gross Observation

The WSSV-infected *Penaeus monodon* collected was gross observed and the length of animals varied from 8.2 and 8.9 cm and the weight 5 to 8.7 g respectively. The samples collected were also observed for clinical symptoms of the infection. The shrimp had visible white spots on the carapace and abdomen which indicated the basic symptom of WSSV infection.

Wet Mount Observation

The carapace with a white spot mounted on the glass slide was viewed under the microscope and found to have the double membraned floral structure which has been reported earlier in WSSV infected samples (Yoganandhan, *et al*., 2003), Hence this wet mount study confirms the spot is caused by WSSV infection (Fig 3).

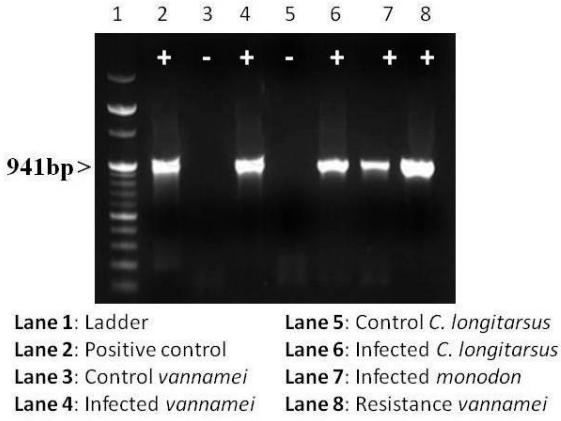
Fig 3: Double membrane floral structure

PCR detection of WSSV in *Penaeus monodon* DNA isolation

The DNA has been isolated from four randomly selected *Penaeus monodon*; a shrimp that was found to be positive for infection already was used as a positive control.

Polymerase Chain Reaction (PCR)

The DNA isolated has been amplified by PCR analysis with the WSSV-specific primers. The results revealed the presence of WSSV infection in *Penaeus monodon* samples. The PCR product size for WSSV is 941bp. Positive controls showed the same molecular weight as sample products and negative control had no bands in electrophoresis (Fig 4).



Lane 1: Ladder
Lane 2: Positive control
Lane 3: Control *vannamei*
Lane 4: Infected *vannamei*
Lane 5: Control *C. longitarsus*
Lane 6: Infected *C. longitarsus*
Lane 7: Infected *monodon*
Lane 8: Resistance *vannamei*

Fig 4: Gel image showing PCR diagnosis of clinically normal and WSSV infected animal tissue. Lane 1, 100bp ladder, Lane 2,4,6,7 & 8 clinically infected sample; Lane 3&5 control negative.

Infectivity Study

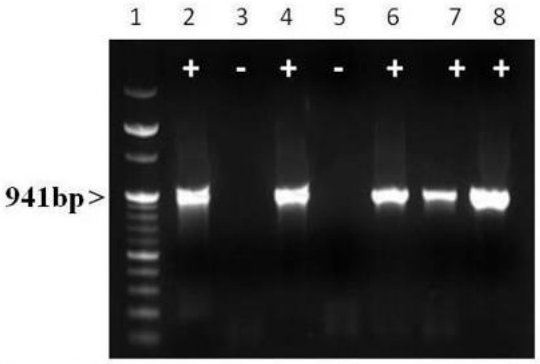
The infectivity was conducted on *L. vannamei* and *Clibanarius longitarsus* Infection was introduced by the modes of ingestion (Fed with infected *Penaeus monodon*). The animals were fed twice daily; in case of control, the animals were fed with commercial pellet feeds. The experimental animals were observed regularly. On the subsequent days of the infectivity study, the infected *Litopenaeus vannamei* showed lethargy and began swimming in the sides. Later on, on the third day of infection mortality started. *Clibanarius longitarsus* Though all the shrimps were dead from the infectivity test, the hermit crab tank was monitored for 30 days and found to be active and normal. On the 30th day, the animals were sacrificed and analyzed for the prevalence of infection.

WSSV detection of experimental animals by PCR DNA isolation

The DNA has been isolated from four randomly selected *L.vannamei* from the infectivity challenge test and two randomly chosen *Clibanarius longitarsus* A shrimp that was found to be positive for infection already was used as a positive control.

Polymerase Chain Reaction (PCR)

The DNA isolated has been amplified by PCR analysis of the WSSV-specific primers. The results revealed the presence of WSSV infection in *L.vannamei* and *Clibanarius longitarsus* samples. The PCR product size for WSSV is 941bp. Positive controls showed the same molecular weight as sample products and negative control had no bands in electrophoresis (Fig 5).



Lane 1: Ladder
Lane 2: Positive control
Lane 3: Control *vannamei*
Lane 4: Infected *vannamei*
Lane 5: Control *C. longitarsus*
Lane 6: Infected *C. longitarsus*
Lane 7: Infected *monodon*
Lane 8: Resistance *vannamei*

Fig 5: Gel image showing PCR diagnosis of clinically normal and WSSV infected animal tissue. Lane 1, 100bp ladder, Lane 2,4,6,7 & 8 clinically infected sample; Lane 3&5 control negative.

SDS-PAGE

Protein was extracted and purified from animal tissue and SDS-PAGE was performed and the bands were observed under GEL-DOC.

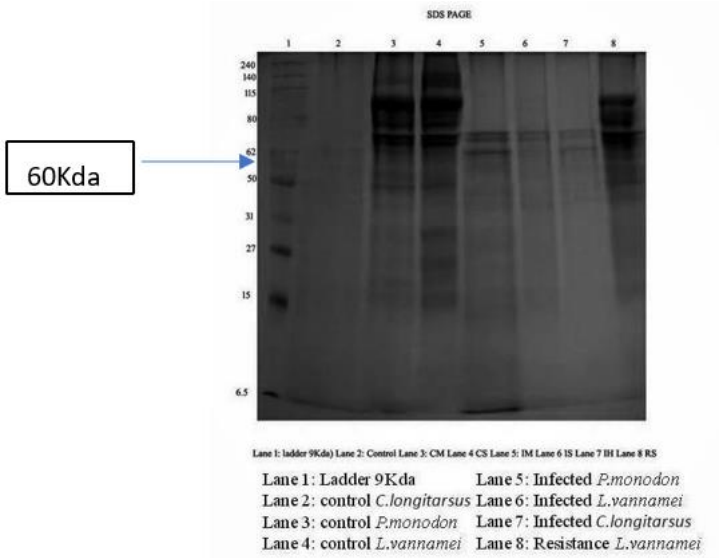


Fig 6: Coomassie-Brillant-Blue-stained SDS-PAGE gel (10%) of WSSV infected and uninfected muscle tissues of study animal Note the presence of expressed protein bands in WSSV infected muscles (lane 5,6,7,8) as compared with uninfected muscles (lane 2,3,4).

From the above image, it is clear that there is a change in protein expression before and after infection. Expression of HSP60 (60Kda) after infection, which is a heat shock protein, also known as a chaperonin. When infected or diseased the animals were found under stress, it naturally produces stress protein such as HSP60. It acts as a danger signal cascade. The heat shock response is a homeostatic mechanism that protects a cell from damage by upregulating the expression of genes that code for HSP60. The upregulation of HSP60 production allows for the maintenance of other cellular processes occurring in the cell, especially under stress (Fig 6).

DISCUSSION

White spot syndrome is a major aquatic disease which is catastrophic to the aquaculture industry. Studies reveal that the prevalence of this viral infection in other crustaceans that are also observed as host and asymptomatic carriers for example, crayfish (Jiravanichpaisal *et al*., 2001), crab (Gunasekaran *et al*., 2018), rotifers (Zhang *et al*., 2010), artemia(Yan *et al*., 2004) and polychaete worms (Vijayan *et al*., 2005).

WSSV is known to get transmitted to cultured shrimp through contaminated water and ingestion of WSSV-infected animals (Supamattaya *et al*., 1998). In both natural and farm environments, the principal mode of disease transmission is oral ingestion as the animals are cannibalistic. Cohabitation also aids in the transmission of infection to shrimp farming system and wild. (Flegel and Alday-Sanz, 1998)

WSSV contaminated phytoplankton can be a vector for WSSV transmission to penaeid shrimp larvae (Zhang *et al*., 2006). In the present study, the detected viral infection in the cytoplasm and nucleus of the gill cells were rod-shaped with a size ranging between 245 to 310 nm in length and 100 to 125 nm in width.

The crabs can tolerate a wide variation in temperature, salinity, pH, and dissolved oxygen. Therefore, they are available in almost all the marine ecosystems. Similar test reports were also available on WSSV infection through ingestion and water-borne mode against *Scylla serrata* (Lo *et al*., 1996). In this study, it was revealed that an increase in viral load in total exchange of WSSV challenged animals within 48 hrs, and later with the profound expression of all clinical symptoms, typically for white spot syndrome (Sanchez-Paz, 2010). Investigations were also showed that the rotifers might be WSSV carriers and act as reservoir for WSSV (Yan *et al*., 2004, 2007). Further, it was reported that the cell membrane of the rotifer, *Brachio nusrceus* known to bind the WSSV (Yan *et al*., 2007).

The present study exhibited that both *Clibanarius longitarsus* (Hermit crab) and *Litopenaeus vannamei* (Shrimp) were proved on the prevalence of WSSV after they were subjected to artificial infection. Further, it was confirmed through SDS PAGE to differentiate the proteins of downregulated or upregulated for pre- and post-infection stages respectively.

CONCLUSION

The gel electrophoresis of SDS PAGE reveals the expression of Heat shock protein (HSP 60) which is a dangerous signal cascade that is expressed as an immune response. This WSSV infectivity study on both *Litopenaeus vannamei* and

Clibanarius longitarsus showed commendable results as the hermit crab was noticed with asymptomatic and healthy when compared to shrimp. Therefore, the efficacy of this study would serve as base-line for potential drug designing as the prevalence of WSSV infection in the hermit crab caused no mortality that has raised interest to proceed further on the evaluation of proteins that involved in protecting the animal from serious health concern.

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