Analysis of white spot syndrome virus (WSSV) consignment in the progeny of specific pathogen free (SPF) Pacific *Litopenaeus vannamei* by RT-PCR using SYBR Green chemistry

P Kavitha, M A Badhul Haq*, R Shalini, A Sajith Ahamed and M Srinivasan
Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai 608 502, India

Received 4 April 2012; revised 18 November 2012; accepted 14 January 2013

A rapid and highly sensitive detection and quantification method for white spot syndrome virus (WSSV) was developed using GeneAmp 5700 Sequencer Detection System and SYBR Green chemistry. The RT-PCR mixture contained the fluorescent dye SYBR Green, which exhibited florescence enhancement upon binding to double strand cDNA. About $10^5$ and $10^6$ copies µg$^{-1}$ of viral DNA load were observed in the juvenile offsprings of shrimps (*Litopenaeus vannamei*) originated from Hawaii (Hawaii A) and Singapore, respectively. The inocula of known virus content, derived from three different place of origin, viz., Hawaii (Hawaii-A), Singapore and Thailand, showed different logs of WSSV copies as $10^7$, $10^6$ and $10^5$ WSSV copies ng$^{-1}$ at DNA, respectively. In the positive control, a higher amount DNA copies were noticed. An appropriate quantity of $10^7$, $10^6$ and $10^5$ WSSV copies of ng$^{-1}$ DNA was observed in the juvenile offsprings of shrimp, respectively originated from Hawaii-A, Singapore and Thailand. More importantly, there was no sign of WSSV load till the end of assay in the entire control group of juvenile offsprings. The least quantity of WSSV load ($10^5$ WSSV copies ng$^{-1}$) was recorded in the juvenile offsprings of *L. vannamei* originated from Thailand.

**Keywords:** WSSV, SPF, *Litopenaeus vannamei*, SYBR Green, offspring’s origin

**Introduction**

Viral diseases are considered to be the single most devastating problem in shrimp culture and have seriously affected the sustainability and economic success of the Indian shrimp aquaculture industry. White spot syndrome virus (WSSV) is the most serious pathogen and threatens heavy loss of the aquaculture industry. WSSV is an enveloped rod-shaped virus, containing double-stranded DNA. It is large (70-150 nm × 275-380 nm) and extremely virulent, causing heavy mortalities, up to 100% within 3-10 days in affected shrimp stocks. Based on the 8th Report of the International Committee on Taxonomy of Viruses (ICTV), WSSV is found to be a species of the genus Whispovirus, a member of the new family Nimaviridae. It contains a single molecule of circular dsDNA with a size of 300 kb. The complete genomic sequence of WSSV has been determined during the past several years. The genome contained approx 180 putative open reading frames (ORFs), of which only a few could be assigned to putative function. Therefore, further studies are needed to fully understand the basic nature of WSSV, its exact life cycle and mode of infection.

The presence of viral genomes in the infected animals has become one of the most important parameters used to monitor the progression of the disease. However, quantification of WSSV has been hampered by the lack of a continuous cell culture system for shrimps. The recent development of the quantitative competitive polymerase chain reaction circumvents this obstacle and provides an attractive tool to quantify the number of WSSV genomes in individual shrimp. Genomic quantification is obtained by comparing the quantity of amplicons of the specific target sequence with the amplicon of the internal standard at known concentrations. This method has been applied in the quantification of many important viruses, such as, human immunodeficiency virus (HIV) and hepatitis B virus (HBV), in monitoring viral replication and response to antiviral treatments.

The present study was made to assess the sensitivity and specificity of SYBR green RT-PCR in detecting WSSV in the progeny of specific pathogen free (SPF) *Litopenaeus vannamei* brooders. As the technology has been adopted rapidly in developing countries in Asia, there is an urgent need to develop an action plan for research and training activities that will facilitate more effective utilization of the technology.

*Author for correspondence:
Tel: +91-4144-243223; Fax: +91-4144-243555
E-mail: drmabhaqcas@gmail.com
Materials and Methods
A total of 30 samples of SPF *L. vannamei* shrimp was collected from the leading shrimp hatchery and farm industry, situated in Chettinagar village and Marakkanam village in Villupuram District, Alappakkam village in Cuddalore district, Perunthuravu village in Kanchipuram District and Koovathur in Ariyalur district of Tamil Nadu, and Kotta in Nellore District and Kothapatname Mandal in Prakasam District of Andhra Pradesh, which were imported from Hawaii (USA), Singapore and Thailand.

Sample Preservation
The tissue samples for DNA extraction were cut into small pieces (< 5-7 mm) to permit adequate fluid penetration and preserved in fresh 95% ethanol using 1.5 mL tubes. The ethanol was 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
Total DNA was extracted from shrimp hemolymph, gills and pleopod tissues by routine High Pure PCR template preparation kit method.

Primer Design
Primers were designed using web-based software, such as, Primer 3 and Primer Express, developed by Primer Design.

Construction of Positive Control Vectors and Standards for Quantification
The standard for the WSSV positive was conformed by the sample from Badhul Haq *et al* and prepared by diluting the DNA sample ~4-fold, made up as dilution of to 21 µL of WSSV DNA sample with 59 µL H₂O for a final volume of 80 µL, which was vortexed, spun down and then transferred with equal quantity from each sample into a single tube. The WSSV positive standard dilution factor and value is listed in Table 1.

<table>
<thead>
<tr>
<th>Standard no.</th>
<th>Dilution factor</th>
<th>Dilutions</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pool</td>
<td>360 µL Standard 1</td>
<td>25600</td>
</tr>
<tr>
<td>2</td>
<td>1:4</td>
<td>90 µL Standard 1+270 µL H₂O</td>
<td>6400</td>
</tr>
<tr>
<td>3</td>
<td>1:16</td>
<td>90 µL Standard 2+270 µL H₂O</td>
<td>1600</td>
</tr>
<tr>
<td>4</td>
<td>1:64</td>
<td>90 µL Standard 3+270 µL H₂O</td>
<td>400</td>
</tr>
<tr>
<td>5</td>
<td>1:256</td>
<td>90 µL Standard 4+270 µL H₂O</td>
<td>100</td>
</tr>
</tbody>
</table>

SYBR Green Assay and PCR Analysis
SYBR specific Green PCR Master Mix was used for the assay. The primer concentrations were normalized and gene-specific forward and reverse primer pairs were mixed. The primer (forward or reverse) concentration in the mixture was maintained at 5 pmol/µL. The fluorescent dye SYBR Green I binds to the minor groove of the DNA double helix. In solution, the unbound dye exhibited very little fluorescence; however, fluorescence was greatly enhanced upon DNA-binding.

A RT-PCR reaction mixture could be either 50 µL or 25 µL. From the SYBR Green kit 25 µL SYBR Green Mix (2×), 0.5 µL liver cDNA, 2 µL primer pair mix (5 pmol/µL each primer), 22.5 µL H₂O was added in each optical tube. Amplification was performed in a thermocycler using the following protocol: 1 cycle at 94°C for 2 min, then 94°C for 20 sec, 62°C for 20 sec, 72°C for 20 sec, repeated 15 cycles, then added 72°C for 30 sec and 20°C for 30 sec at the end of the final cycle. After PCR, the specificity was examined on 1.5% agarose gel using 5 µL from each reaction. The PCR results were analyzed with the SDS 7000 software.

Results and Discussion
The SYBR Green RT-PCR assay was carried out for quantification of the WSSV genome. This method is based on competitive co-amplification of a target WSSV genome together with known concentrations of an internal standard in one-tube reaction. The data indicated that there was a positive correlation between high viral load and severity of disease as usually determined by quantitative PCR. The concentrations of SYBR Green I above 0.7× were inhibitory to PCR as evidenced by the delay in Ct value. This was expected based on previous reports in the literature. In the present study, the cumulative WSSV mortality of SPF *L. vannamei* was observed due to the pathogenicity in captivity.

The analytical sensitivity of SYBR Green PCR was performed by using a serial dilution of WSSV DNA as template for amplification. A wide range of 0-20 copies µg⁻¹ at DNA were found in the juvenile offsprings of *L. vannamei* brooders, imported from Hawaii (Hawaii-A), Singapore and Thailand. In the present study, a positive standard used in the range of 10², 10³, 10⁴, 10⁵ and 10⁶ copies of DNA in all the above WSSV infected juveniles was noticed. Quantification to the moribund offsprings with the average body weight of 6.8 g of Hawaii A, Singapore and Thailand.
KAVITHA et al.: DETECTION OF WSSV BY SYBR GREEN RT-PCR

and Thailand were collected and were quantified for SYBR PCR assay in one offspring and the consequences of WSSV load was calculated as 0.000001 copies µg⁻¹ of total DNA in the cephalothoraxes region of juveniles. In addition, 10⁷ copies ng⁻¹ at DNA were observed in the offsprings of Hawaii A originated juveniles. Similarly, the DNA load in the Thailand originated brooders was encountered as 10⁶ copies ng⁻¹ and this trend was found uniform in severe and acute cases of juveniles infected with WSSV. However, the inoculum of known virus content, bioassayed by three different above originated samples showed in different logs of WSSV copies as 10⁷, 10⁶ and 10⁵ WSSV copies ng⁻¹ at DNA. In the positive control, a higher amount of WSSV load was observed as copies. The WSSV load using SYBR green RT-PCR were analyzed in the tissues, such as, lymphoid organ, hematopoietic organ and gill lamellae, from the above 3 different offsprings (juveniles) spawned by the brooders imported from Hawaii (Hawaii A), Singapore and Thailand.

The mean viral copy number per nanogram of total DNA (× cn/ng tDNA) extracted ranged from 10⁷, 10⁶ and 10⁵. In the Hawaii A (USA), Singapore and Thailand origins, the × cn/ng of extracted tDNA ranged from 0 to 0.10⁵ in the shrimp head. When these values were converted to mean viral copy number per gram (× cn/g) of tissue, the values increased in range. In a previous study, the WSSV infections in three broodstock showed a wide range, from 0 to 2.28×10⁶ (with a mean of 1.50×10⁵) copies ng⁻¹ of DNA. Of 159 brooders assayed, 39 (24.5%) were negative and 120 (75.5%) were positive; 153 (96.2%) showed less than 100 copies (mean 10.2 copies), 111 (69.8%) exhibited less than 10 copies, and only 6 individuals (3.8%) expressed high infections with a range of 2.36×10² to 2.28×10⁶ copies ng⁻¹ of DNA. In 210 post larvae, a range of 2.6 to 713.6 (with a mean of 220) copies ng⁻¹ of DNA had been observed. There has been limited information concerning SYBR Green RT-PCR quantification of WSSV viral genome studies in the penaeid shrimps of the Indian waters, particularly SPF Pacific white shrimp L. vannamei.

Real-time PCR results with SYBR Green I concentration ranged from 0.2 to 0.7×. The positive standard was used in the range of 10², 10³, 10⁴, 10⁵ and 10⁶ copies ng⁻¹ of DNA. Sample 1 recorded approx 10⁷ WSSV copies ng⁻¹ of DNA in the WSSV infected juveniles of brooders imported from Hawaii A, USA (Fig. 1). The consequences were recorded approx 10⁶ WSSV copies ng⁻¹ of DNA as detected in the offsprings of WSSV infected brooders, imported from Singapore (Fig. 2). However, very low level infectivity of approx 10⁵ copies ng⁻¹ of WSSV DNA was noticed in the offsprings of brooders imported from Thailand (Fig. 3). Moreover, there was no evidence of WSSV viral copies observed in the control samples, placed for the present investigation.
An approx 20 copies of WSSV ng$^{-1}$ of DNA was detected in the positive control of WSSV infected *L. vannamei* juveniles evaluated for this assay (Fig. 5; Table 2). Similar results have been demonstrated in a previous study where linear range of the standard curve of the WSSV VP28 fragment was generated over a range of 7 log units. The upper and lower quantification limits were $1.24 \times 10^7$ and 12 copies per PCR reaction, respectively$^{12}$. Thus, the present results indicate that the imported SPF brood stock spawned culture shrimp *L. vannamei* and native culture shrimp *P. monodon* obtained from natural Indian waters might be infected with WSSV and had the potential of spreading into the SPF *L. vannamei* farming environment.

**Acknowledgement**

Authors are greatly indebted to the shrimp farmers of Tamil Nadu and Andhra Pradesh region and the authorities of Annamalai University for providing support and encouragement throughout the study period. The University Grants Commission [F. No: 41-4/2012 (SR)] and the Department of Science and Technology [SERB Fast Track Scheme No.: SR/FT/LS-125/2011]], Government of India, New Delhi are also acknowledged for providing the financial assistance.
References