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Research Article

GEOGRAPHICAL DIFFERENTIATION AND WSSV INFESTATION OF SPF *LITOPENAEUS VANNAMEI* BROOD STOCK SHRIMP USING MOLECULAR VERDICTS

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ABSTRACT

Viral diseases are considered to be the single most devastating problem in shrimp culture and have seriously impacted the sustainability and economic success of the Indian shrimp aquaculture industry. Intensive research is still required to fully understand the basic nature of WSSV, its exact life cycle and mode of infection. The molecular phylogenetic analysis of SPF *Litopenaeus vannamei* broodstock (genetically feasible) that were collected from Tamil Nadu and Andhra Pradesh eateries were imported from different province of the world to the Indian shrimp hatcheries are presented: 1) Hawaii-B 2) Florida 3) Hawaii- A 4) Singapore and 5) Thailand. Next to the Hawaii- B a high genetic variability showed in the *L.vannamei* broodstock imported from Florida. The WSSV genes were collected from *L. vannamei* in two different origins from two south Indian shrimp hatchery zones. The RT-PCR mixture contained fluorescent dye, SYBR Green chemistry assay, which exhibits fluorescence enhancement upon binding to double strand cDNA. There were about positive of white spot syndrome virus were detected in the Hawaii A originated broodstock and similarly the viral DNA load in the Singapore originated broodstock was encountered as severe positive and this trend was found uniform in severe and acute cases of SPF *L.vannamei* broodstock shrimp infected with WSSV, which were collected from Tamil Nadu Andhra and Pradesh hatchery premises.

Keywords: *Litopenaeus vannamei,* SPF, broodstock, WSSV, SYBR Green chemistry assay

INTRODUCTION

Shrimp culture is the primary contributor to the coastal aquaculture all over the world. The potential brackish water area available in coastal regions of country for shrimp culture is about 1.2 million hectares is under culture. The export of cultured shrimps from our country is about 50% of the total shrimp exports. Hence there is a enormous potential for further expansion of coastal shrimp aquaculture in our country. But the rapid increase in shrimp farming in the last decade has resulted in environmental and social concerns. However these concerns are now largely taken care of and the social acceptability of shrimp farming has been attained. The recent globalization in trading the products has brought in many new issues such as traceability of the produce, stringent quality profiling of the aquaculture from food safety angle, especially residues of antibiotics, heavy metals and pesticides, disease transmission, etc. all of which call for a sound regulatory framework for this sector and the various programmes undertaken by Coastal Aquaculture Authority (CAA) adequately address these problems (Anonyms, 2010). Viruses are considered to be the most important pathogens in shrimp have caused severe production and economic losses in the past two decades (Haq *et al* 2013). More than 20 viruses have been reported as pathogenic to shrimp (Haq *et al* 2012).

White spot syndrome (WSS) continues to be one of the most serious disease problems faced by the shrimp farming industry not just in Asia and globally (Takahashi *et al.,* 1994; Chou *et al.,* 1995; Wongteerasupaya *et al.,* 1995; Lo *et al.,* 1996a, b; Flegel 1997; Karunasagar *et al.,* 1997a; Hsu *et al.,* 1999; Haq *et al* 2011). WSSV has the potential to infect cultured shrimp and incur a mortality rate of 100 percent of the population within 3 to 10 days. The white-spot baculovirus is a large, enveloped, and Rod-shaped to somewhat elliptical non-occluded virus consisting of double-stranded DNA.

The WSSV is extremely virulent, and it attacks primarily tissue cells, including ectodermic and mesodermal tissues, connective tissues of organs, nervous tissues, muscle, lymphoids, and hematopoietic tissue, stomach, gills, antennal glands, heart, and eyes are also affected; resulting in the total lysis of the cells. WSSV belongs to a new virus family known as Nimaviridae. Its genome is about 300,000 base pairs in length, based on analysis of different isolates. The virions contain one nucleocapsid with 5 major proteins and at least 13 minor proteins (Witeveldt *et al*., 2004).

WSSV is a bacilliform, non-occluded enveloped virus (Chou *et al*. 1995; Wang *et al*. 1995; Wongteerasupaya et al. 1995). Intact enveloped virions range between 210 and 380 nm in length and 70–167 nm in maximum width (Flegel & Alday-Sanz 1998). The viral envelope is 6–7 nm thick and is a lipidic, trilaminar membranous structure with two electron-transparent layers divided by an electronopaque layer (Wongteerasupaya *et al*. 1995; Durand 1997).

mDNA, Cytochrome Oxidase-1 (CO1) gene sequences were analyzed for species identification and phylogenetic relationship among the very high food value and commercially important SPF Pacific White

shrimp *L.vannamei* species. Sequence analysis of COI gene very clearly indicated that the entire *L.vannamei* animal fell into five distinct groups, which are genetically distant from each other and exhibited identical phylogenetic reservation. All the COI gene sequences from five origin shrimps provide sufficient phylogenetic information and evolutionary relationship to distinguish the *L.vannamei* species unambiguously. This study proves the utility of mtDNA COI gene sequence based approach in identifying best stock *L.vannamei* shrimp at a faster pace.

The PCR method can be a powerful tool for identifying larval forms of an organism and even for incomplete specimens on which a morphological diagnosis cannot be performed (Haq *et al*., 2012). DNA-based detection and diagnostic methods have the potential for widespread application of in aquaculture. As the technology is already being adopted rapidly in developing countries in Asia, there is an urgent need to address these issues and to develop an action plan for research and training activities that will facilitate more effective utilization. As the name suggests, real time PCR is a technique used to monitor the progress of a PCR reaction in real time. At the same time, a relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (*i.e*. SYBR® Green) or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes). Real time PCR facilitates the monitoring of the reaction as it progresses. One can start with minimal amounts of nucleic acid and quantify the end product accurately. Moreover, there is no need for the post PCR processing which saves the resources and the time. These advantages of the fluorescence based real time PCR technique have completely revolutionized the approach to PCR-based quantification of DNA and RNA. Real time PCR assays are now easy to perform, have high sensitivity, more specificity, and provide scope for automation. Real time PCR is also referred to as **real time RT PCR** which has the additional cycle of reverse transcription that leads to formation of a DNA molecule from a RNA molecule. This is done because RNA is less stable as compared to DNA. Real-time PCR, also known as kinetic PCR, qPCR, qRT-PCR and RT-qPCR, is quantitative PCR method for the determination of cope number of PCR templates such as DNA or cDNA in a PCR reaction. There are two flavors of real-time PCR: probe-based and intercalator-based. Both methods require a special thermocycler equipped with a sensitive camera that monitors the fluorescence in each well of the 96-well plate at frequent intervals during the PCR Reaction. Probe-based real-time PCR, also known as TaqMan PCR, requires a pair of PCR primers as regular PCR does, an additional fluorogenic probe which is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. Intercalator-based method, also known as SYBR Green method, requires a double-stranded DNA dye in the PCR reaction which binds to newly synthesized double-stranded DNA and gives fluorescence. TaqMan method is more accurate and reliable than SYBR green method, but also more expensive.

MATERIALS AND METHODS

Collection of *L. vannamei* broodstock sample:

The broodstock shrimps were received from different province of the world namely Hawaii A (Waimanalo) USA), Florida (Islamorada) USA, Thailand (Bangkok), Singapore and Hawaii B (Kailua-Kona). All samples were imported from above mentioned geographical areas; it has been transported to the Indian hatcheries. A total of 300 numbers of male and female *L.vannamei* brooders were collected from twelve different locations (12 Indian hatcheries), *i.e.* fifty prawns from each viz., shrimp hatchery of both Tamil Nadu and Andhra Pradesh coastal waters are as follows.

Average body weight (ABW) of 40 – 60 g of SPF *L.vannamei* brooders were collected from different hatcheries of different origin located in the Tamil Nadu and Andhra Pradesh hatcheries of India and the shrimps were transported in the plastic containers to the marine virology laboratory. Upon arrival to the laboratory the brooders were ice killed and were stored at -20˚C for further molecular diagnosis.

The composition of samples is multi location, signifying the characteristic feature of the origin. All the shrimps were identified morphometrically, with the help of FAO-Fisheries identification sheets. The *L.vannamei* brooders collected from five different genesis were based on the destinations, as origin 1 (Hawaii A USA), origin 2 (Florida USA), origin 3 (Singapore), origin 4 (Thailand) and origin 5 (Hawaii B USA) based on their geographical distribution. Most of Indian hatchery were imported Hawaii and Florida based origin location.

Isolation of genomic DNA:

Genomic DNA was followed by Haq *et al*., (2012) method, briefly described below: Salting out procedure was adapted to extract DNA from *L.vannamei* tissues. The preserved tissue in ethanol was washed four to five times with sterile distilled water to get clear of the ethanol content. The ethanol free tissues was transferred in to 1.5 ml tube and grounded in micro pestle with 500µl of solution 1 (500mM Tris-HCL, 20mM EDTA and 2% SDS). After homogenizing the tissues were added with 5µl of Proteinase K (20mg/ml).

The tubes were incubated at 55ºC in water bath for 2 hours with occasional mixing by inverting the tubes. Following incubation the samples were chilled on ice for 10 minutes and about 250µl of solution 2 (6M NaCl) was added to it and mixed well by inverting the tubes several times. Tubes were then chilled on ice for 5 minutes. Then the tubes were centrifuged at 8000 rpm for 15 minutes and following centrifugation, 500 µl of clear supernatant was collected in a 1.5 ml tube. Equal volume of (1ml) of 100% analytical grade ethanol was added to precipitate the DNA. A thin hair like precipitate was observed after addition of ethanol. After 30 minutes the tubes were allowed to spin at 11,000rpm for 5 minutes. The supernatant was removed and partially dried in room temperature. The DNA pellets were washed thrice with 70% cold ethanol. The pellets

were suspended in 100 µl of sterile distilled H2O.

Sequencing analysis:

Nucleotide sequencing was performed using the di-deoxy termination method of Sanger modified by Chen and Seeburg. PCR product was used for sequencing after precipitation. Big Dye terminator cycle sequencing kit (Perkin Elmer) provided the reaction mix containing all the dNTP's and the differentially labelled four deoxynucleotides along with Taq DNA Polymerase in the sequencing buffer. The processed sequencing microtiter plate was loaded on an automated DNA sequencer (Applied Biosystems, model 3700) for sequencing. The sequencing was done both in the forward and reverse directions.

Real-time PCR analysis:

PCR technique has been commonly employed in the aquaculture industry for disease diagnosis and prevention. With the growing demand for a quantitative research tool and requirements for a high throughput screening, real time PCR is receiving more attention and becoming more important in the industry.

SYBR Green chemistry assay has developed quantitative system, a real time based diagnosis system. It not only meets the demands, but its advanced designs. Listed below, also provides a more accurate, more sensitive and more flexible real- time system to the shrimp farming industry.

SYBR Green Assay:

SYBR specific Green PCR Master Mix:

Obtain superior sensitivity and reproducibility without compromising specificity, dynamic range or uniformity in your real-time, quantitative PCR experiments. SYBR® Green PCR Master Mix and RT-PCR Reagents deliver highly sensitive DNA, cDNA and RNA quantitation, detecting as few as 2 copies of a target gene over a broad range of template concentrations. The SYBR Green PCR Master Mix offers significantly improved sensitivity by employing a highly purified AmpliTaq Gold® DNA Polymerase, LD in a newly optimized formulation. Our newly optimized formulation ensures consistent results. In addition, the new SYBR Green PCR Master Mix replaces the SYBR Green PCR Master Mix in existing Applied Biosystems protocols using the same reaction preparation and thermal cycling conditions. Normalize the primer concentrations and mix gene-specific forward and reverse primer pair. Each primer (forward or reverse) concentration in the mixture is 5 pmol/µl.

SYBR Green Kit Assay:

Make 12µl of master mix for each well, plus some excess

1 Rxn:	10µl SYBR Green Mix	52 Rxn:	520µl SYBR
	0.4µl Forward Primer (10µM stock)		20.8µl F
	0.4µl Reverse Primer (10µM stock)		20.8µl R
	1.2µl H_2O		62.4µl H_2O
	12µl per well (+ 8µl cDNA = 20µl final volume)		624µl

2. A real-time PCR reaction mixture can be either 50 µl or 25 µl. Prepare the following mixture in each optical tube from the SYBR Green kit:

25 µl SYBR Green Mix (2x)		12.5 µl SYBR Green Mix (2x)
0.5 µl liver cDNA		0.2 µl liver cDNA
2 µl primer pair mix (5 pmol/µl each primer)	OR	1 µl primer pair mix (5 pmol/µl each primer)
22.5 µl H_2O		11.3 µl H_2O

After PCR was finished, removed the tubes from the machine. The PCR specificity was examined by 3% agarose gel using 5 µl from each reaction. Put the tubes back in SDS 7000 and perform dissociation curve analysis with the saved copy of the setup file. Analyze the real-time PCR result with the SDS 7000 software. Check to see if there is any bimodal dissociation curve or abnormal amplification plot.

Construction of positive control vectors and standards for quantification:

Making the Standards:

Every gene can run on qPCR were needed to run with a standard curve in order to relatively quantitate the Ct values of respective samples. The following protocol assumes that has been created cDNA from DNA prior to qPCR. If followed the Invitrogen SuperScript III kit's protocol were started with 21µl of cDNA per sample.

Diluted each cDNA sample ~4-fold. In this case, diluted 21µl with 59µl H_2O for a final volume of 80µl. Vortex and spin down. Transferred equal amount from each sample into a single tube. This was standard 1, high standard. To determined how much to pull from each sample, calculate how much were left in each sample and what the final volume of respective standards. (This is to have approximately the same final volume of standards and samples.)

Example:

Taken 30µl from each of 12 samples and poured for standard 1 with a final volume of 360µl.

Final sample volume have been sample volume * 5 after a five-fold dilution (see below). In this case, (80µl - 30µl) * 5 = 250µl of each sample, final volume.

Taken 90µl of standard 1 in a new tube labeled standard 2. Diluted standard 2 with 270µl H_2O for a final volume of 360µl. Repeated up to Standard 5.

Standard Number	Dilution Factor	Dilutions	Value
1	P	360µl Standard 1	25600
2	1:4	90µl Stnd. 1 + 270µl H_2O	6400
3	1:16	90µl Stnd. 2 + 270µl H_2O	1600
4	1:64	90µl Stnd. 3 + 270µl H_2O	400
5	1:256	90µl Stnd. 4 + 270µl H_2O	100

Table 1: WSSV positive standard dilution factor and value

Real-time PCR amplification:

Running the qPCR:

Quantitative PCR requires that a certain type of machine capable of detecting SYBR fluorescence while performed PCR was used.

Note: The PCR programme were design all the primers to ideally function at about 60°C. Therefore, PCR conditions resemble the following:

94°C – 15 minutes (for the Qiagen mix mentioned earlier)

94°C – 15 sec.
60°C – 30 sec. } 35 cycles
72°C – 30 sec.

Data analysis:

Baseline and background fluorescence:

As already known, the utilization of fluorophore(s) in the reaction allows us to track and monitor the dynamics of the reaction via the fluorescence signals given out by the fluorophore. In addition, under normal

conditions a fluorophore can give out a low level of fluorescence signal that has no relevance to the dynamics of the qPCR even though the fluorophore was not excited by any light source. This low level of fluorescence signal was described as background signal. This background signal contributes to part of the baseline fluorescence of the reaction. The baseline fluorescence were the noise level in early PCR cycles, typically measured between cycles 3 and 15, where there was no detectable increased in fluorescence due to amplification products. Because of this, the "noise" from the baseline fluorescence must be deducted from the total fluorescence signal output or "raw fluorescence (R)" as it was denoted after the reaction so that the quantitative result was more meaningful. This deducted value was described as "baseline corrected fluorescence (DR)". If a passive reference dye is used, its fluorescent intensity (Rrox) is used to normalize the baseline fluorescent and the term normalized baseline (DRn)" is used.

Threshold line and Quantification cycle:

The threshold line was considered as a boundary that separates the reaction's detection signal from the background signal. This line was calculated as the average baseline plus 10 times the standard deviation of that baseline in all real-time PCR machines available on the market. This line often manually set by the user to the early log phase of the reaction when there was a significant fluorescence signal detected in relation to the baseline fluorescence signal. Now during the exponential amplification of target, the fluorescent signal cross the threshold line at a specific cycle and this is called quantification cycle (Cq) or threshold cycle (Ct). In other words, the Cq value was the cycle number whereby the reaction goes into the exponential phase and its fluorescence signal exceeds the background fluorescence signal.

RESULT

Geographical Differentiation:

The standard nucleotide composition between different populations was compared between the populations, using the equations formulated by the Tamura *et al.* (2011) for the molecular evolutional genetic analysis using maximum likelihood assay. A maximum G+C content of 40.2 % and a minimum of 38.1 % were noticed in the *L.vannamei* broodstock originated from Thailand and Hawaii - A respectively however, the level of an important elemental significant of the test organism viz., A+T found as a highest of 61.9 % and lowest of 59.8% in both the *L.vannamei* broodstock originated from the Hawaii B and Thailand respectively. Molar concentrations were estimated to the *L.vannamei* belonging to six different geneses were observed between the base pairs.

Average nucleotide number in molar concentration in each origin was presented as: Hawaii-A, USA (179) > Singapore (179) > Florida, USA (178.5) > Hawaii-B, USA (178) > Thailand (142.4). However, a similar average level between base pair level was presented as in thiamine 245 > cytosine 143.6 > adenine 191.6 >

glycine 133.6. Contrarily, the higher level percentage base pairs in *L.vannamei* brooders found in thiamine (31.34%) correspondingly, an average uniformity in the molar concentration between different origin showed 25% and the DNA nucleotides in COI region of *L.vannamei* broodstock are showed below.

Name of Origin	Accession ID	Base pair length	G+C content (%)	A+T content (%)	Nucleotide Number and Mol%			
					A	T	G	C
Hawaii-A	JN165700	716	38.1%	61.9%	193 27.0 %	250 34.9 %	132 18.4 %	141 19.7%
Florida	JN165701	714	38.5%	61.5%	194 27.2 %	245 34.3 %	134 18.8 %	141 19.7%
Singapore	JN165702	716	38.3%	61.7%	193 27.0 %	249 34.8 %	131 18.3 %	143 20.0%
Thailand	JN165703	712	40.2%	59.8%	190 26.7 %	236 33.1 %	137 19.2 %	149 20.9%
Hawaii-B	JN165704	712	39.0%	61.0%	188 26.4 %	246 34.6 %	134 18.8 %	144 20.2%

Table 2: Nucleotide composition of test organisms

Representing the molar concentration of DNA nucleotides in the COI region of *L. vannamei* samples (Origin 1- 5).

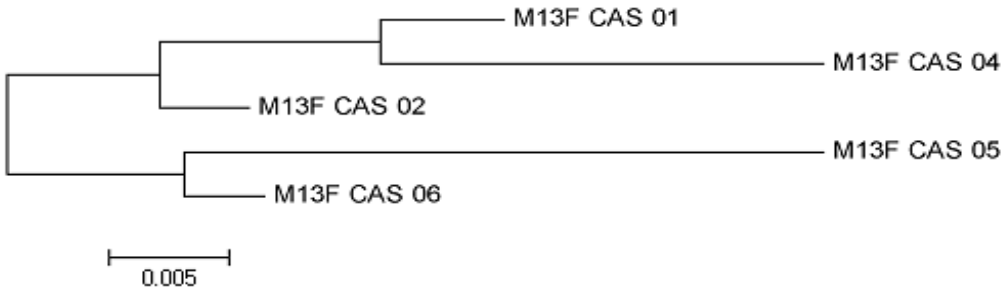

Figure 1: Molecular Phylogenetic analysis by Maximum Likelihood method.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1301.8479) is showed. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 5 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 708 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura, *et al*. 2011).

Real time PCR Detection of WSSV in *L.vannamei* shrimp broodstock by using using:

SYBR Green Chemistry assay collected from Tamil Nadu and Andhra Pradesh hatchery:

The analytical sensitivity of SYBR Green chemistry RT-PCR was performed by using a serial dilution of WSSV plasmid DNA as template for amplification. Wide ranges of detection level were found in the broodstock *L.vannamei* in Tamil Nadu and Andhra Pradesh hatcheries respectively. In the present study, a positive standard used in the range of 10^2,10^4,10^6, 10^8 and 10^{10} copies of DNA was used as control for this analysis. Detection with amplification plot were observed for SYBR PCR assay in one and the consequences of WSSV level of infection was detected from the total DNA in the cephalothoraxes region of broodstock.

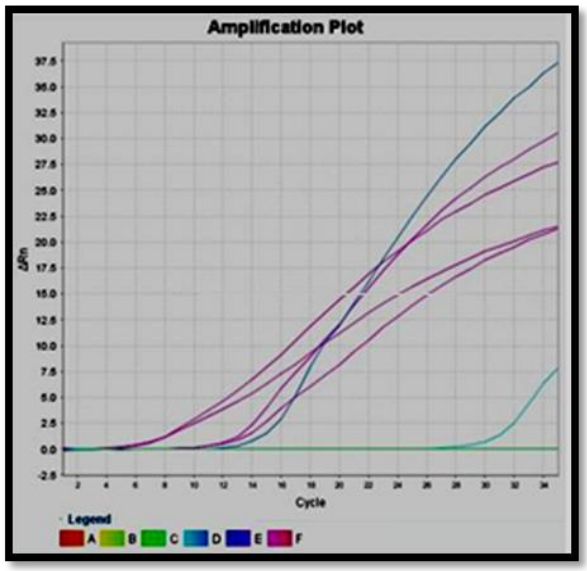

Figure 2: WSSV detection with aamplification plot of *L.vannamei* brood stock collected from Tamil Nadu hatchery

Amplification Plot of Brood stock collected from Tamil Nadu hatchery:

The amplification temperature was performed whole cycles viz., 94°C, 60°C and 72°C respectively.

Total cycling parameters were completed 35 in different amplification steps. The amplification plot was compared with fluorescence intensity in different value. The WSSV PCR product sample with positive standard was amplified uniformly in beneath identical standard copies.

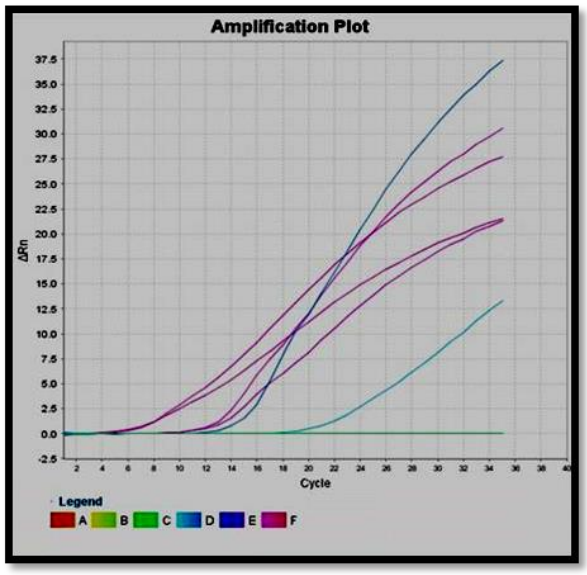

Figure 3: WSSV detection with aamplification plot of *L.vannamei* brood stock collected from Andhra Pradesh hatchery

Amplification Plot of Brood stock collected from Andhra Pradesh hatchery:

The amplification temperature was performed whole cycles viz., 94°C, 60°C and 72°C respectively. Total cycling parameters were completed 35 in different amplification steps. The amplification plot was compared with fluorescence intensity in different value. The WSSV PCR product sample with positive standard was amplified uniformly in beneath identical standard copies.

DISCUSSION

During the last two decades, a combination of poor management practices and intensive culturing of penaeid shrimp has led to the outbreak of several viral diseases. WSSV is one of the most devastating and it can cause massive death in cultured shrimp. Following its first appearance in 1992–1993 in Asia, this disease spread globally and caused serious economic losses. The causative agent of WSD is WSSV, which is a large, non-occluded, enveloped, rod- or elliptical-shaped, dsDNA virus of approximately 300 kbp.

The scientific exploitation and conservation of commercially important fish and shell fish resources is of fundamental importance to any nation. Naturally a basic question arises: what is the natural unit of the

resources that is to be recognised, managed and conserved so as to enable it to replenish while it is continuously exploited? An accurate answer to that question is also of basic importance to planning successful aquaculture breeding programmes (Lester 1992). Sincere efforts to answer to the above question have been initiated many decades ago (Schmidt, 1909). However, different forms of scientific definitions for the units of the resources to be managed and conserved were all established during some recent international symposia held on the topic (deLigny, 1972). The major themes discussed in these two symposia were concerning the units of fishery resources to be managed / conserved and various methods for its identification. A significant outcome of these two symposia was that the ultimate unit of populations of fishery resources to be conserved and exploited is the one that is genetically discrete. The basic and significant role of population genetics in the concept of unit stock structure of fishery resources was highlighted by Altkhov (1981). Genetically, it may be defined as a discrete population which has its own gene pool significantly different from that of other populations (Mangaly, 1974). An important outcome of these two symposia was the comparative evaluation of various resources.

The present study has proved a high frequency of WSSV by RT PCR in both the brood stocks from Tamil Nadu and Andhra Pradesh. The WSSV prevalence was also reported to be quite high in other SPF *L. vannamei* animal in native species *P. monodon* through water contamination, collected in nearby *P. monodon* shrimp farms. Furthermore, a pilot studies of *L.vannamei* from WSSV impact areas of Tamil Nadu coastal waters. Besides all the above factors, the environmental factors may play a key role on the severity of disease outbreaks of aquatic animals. Water temperature, salinity, dissolved oxygen, ammonia, pH and toxins derived from pesticides might be associated with mass mortalities due to WSSV. In SYBR Green RT-PCR, a sample is considered positive when the amplification plot crosses the threshold value. A significant amplification plot of WSSV infected juvenile offspring's of brooders originated from Singapore exceeds threshold value. Whereas amplification plot of juveniles offspring's brooders imported from Thailand did not exceed the threshold line. In order to ensure that the amplification plot the WSSV infected sample was confirmed twice with the specific product, the dissociation curve was analysed. The WSSV amplicon provided dissociation curve as single peak at 72°C in all the three juvenile off spring's of brooders originated from Hawaii A, Singapore and Thailand which is expected for the WSSV specific amplicon. The SYBR Green RT-PCR was not only highly sensitive but also very specific for detecting WSSV and the internal control genes such as EF-1α and β-actin of TSV, YHV. The specificity of SYBR Green RT-PCR, samples is considered positive when amplification plot crosses threshold value. In the present study the amplification plot was compared with fluorescence intensity in different values and WSSV PCR product with positive standard was amplified uniformly in all the cases of brooders imported from Hawaii A, Singapore and Thailand. The RT-PCR consequences interrupted between the offspring's of the Hawaii A and Thailand originated brooders performed, a wide range of 0-20 copies, 0.02 copies μg^{-1} of total DNA resembles Dhar *et al*. (2002) whereas the amplification plot of healthy juveniles didn't excide the threshold line. In SYBR Green RT-PCR, it takes 40 cycles (CT) to detect a single copy of viral

Detection of viruses is over such a large dynamic range is useful for measuring viral load in animals with different levels of infection. Thus SYBR- Green RT-PCR provides a continuous scale measuring the viral load. In addition, since SYBR green RT-PCR is capable of detecting a single copy of viral genome, it will be useful to detect subclinical infections. Due to exquisite sensitivity of SYBR Green PCR, it is highly susceptible to PCR carry over other contamination. Therefore, laboratory practices should be followed strictly to prevent any potential contamination that may give falls positive result. However, any negative result as well as tissues of *L.vannamei* offspring's with CT closed to 20 should be at least twice for conformation. Dhar *et al*. 2002 emphasized the linear relationship between the input plasmid DNA and CT values absorbed from 10^6 down to a single copy of both TSV and YHV. Detection of viruses over such a large dynamic range is used for measuring viral load in animals with different levels of infection.

These results indicate that wild broodstock and native culture shrimp *P. monodon* obtained from natural Indian waters may be infected with WSSV and bring it into the SPF *L. vannamei* farming environment. RT PCR method of detection is potential and will have widespread application in aquaculture. There is an urgent need to address and develop molecular based viral genome technique to save the aquaculture environments. Based on temporal expression profiles, WSSV genes can be classified as early or late genes through RT PCR technique, and they are regulated as coordinated cascades under the control of different promoters. The analyses reveal the uniqueness of WSSV infection range in the region of WSSV outbreak shrimp farms.

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