Journal of Bacteriology and Parasitology

Venkatesan, et al., J Bacteriol Parasitol 2014, 5:5
DOI: 10.4172/2155-9597.1000205

Research Article Open Access

Analysis of Immune Genes and Heat Shock Protein Genes under Exposure to White Spot Syndrome Virus (WSSV) and Herbal Immune Stimulant in *Litopenaeus vannamei*

Venkatesan C[1*], Sahul Hameed AS[1], N Sundarraj[1], T Rajkumar[1] and G Balasubramanian[2]

[1]*Department of Zoology, C. Abdul Hakeem College, Melvisharam, Vellore, Tamil Nadu, India*

[2]*Arinar anna govt arts college, Department of Zoology, Cheyyar, Tamil Nadu*

[*]**Corresponding author:** Venaktesan Chinnakulandhai, Department of Zoology, C. Abdul Hakeem College, Melvisharam, Vellore, Tamil Nadu, India, Tel: +91-416-269487; E-mail: venkatesh3579@gmail.com

Rec date: July 15, 2014; **Acc date:** October 29, 2014; **Pub date:** November 01, 2014

Abstract

This work was carried out to analysis of immune genes (Lectin (245 bp), PoPO (121 bp), BGBP (166 bp), hemocyanin (242 bp), Toll receptor (150 bp) and immunological analysis in white shrimp (*Litopenaeus vannamei*) infected with WSSV and herbal immune stimulant (immuzone) treated. And also to understand the level of expression and distribution of Heat Shock Proteins (Hsp) in white shrimp (*Litopenaeus vannamei*). Under untreated condition, all the immune genes (Lectin, PoPO, BGBP, hemocyanin, Toll receptor) were differentially expressed in all of the examined tissues. Under WSSV infected and immuzone treated condition, immune genes were inducible in all tissues when compared to its untreated condition. The expression levels of Hsp21, Hsp70 and Hsp90 were determined by quantitative real-time PCR in four tissues (gill, hepatopancreas, pleopod and muscle) of *Litopenaeus vannamei* in WSSV treated and normal shrimps. Under untreated condition, all three Hsp genes were differentially expressed in all of the examined tissues. Under WSSV infected condition, only Hsp70 was inducible in all tissues when compared to its untreated condition. The time course induction experiment in gill, muscle, pleopod and hepatopancreas revealed that the transcriptional level of Hsp70 was induced and that Hsp21 and Hsp90 were uninducible under the WSSV treated condition. The expression level of Hsp70 was significantly increased after a 24, 48-h exposure to WSSV whereas the Hsp21 and Hsp90 transcripts were down regulated later after WSSV exposure. This evidence suggests that there is a putative role and involvement of the Hsp genes as a part of immunity response against WSSV in *Litopenaeus vannamei*.

Keywords: Heat shock protein; Gene expression; *Litopenaeus vannamei*; WSSV; Real Time PCR

Introduction

Aquaculture industry is one of the major economic resources of many countries. For the last three decades it is being threatened by many pathogens, especially viruses. Among these viruses, the White Spot Syndrome Virus (WSSV) is the most serious one. The mechanism of WSSV infection and it's host responses induced by WSSV infection remain unclear, Immune gene expression in response to pathogens is of prime importance to understand the immune capability of shrimp and also for the establishment of a health monitoring system in shrimp culture. The molecular mechanisms underlying the majority of antiviral responses in shrimps are still unknown and are only beginning to be addressed [1]. Shrimp molecular responses to viral pathogens have been reviewed by Flegel and Sritunyalucksana [2] as innate immune response comprising the humoral interactions (Toll pathway, proPO and Clotting system, Antioxidant enzymes, Lectins, Haemocyanin, etc.) and the cellular responses (Apoptosis pathway, Jak-STAT pathway, RNAi pathway). Studies to investigate the host immune response after WSSV infection and prophylactic/therapeutic methods have become necessary [3-6]. WSSV structural proteins such as VP19, VP28 and VP26 have been adopted to develop a prophylactic vaccine against WSSV disease [7,8]. Investigate on the molecules and their mechanism relevant to the immune response is fundamental work.

Recently, a series of Heat Shock Proteins (Hsps) were identified; while widely annotating the Expressed Sequencing Tag (EST) databases established from many crustaceans. Hsps are highly conserved proteins which have received the most attention on transcriptional regulation, evolution and innate immune response in recent years [9-11].

Response to a variety of stresses such as temperature extremes, xenobiotics, heavy metals, UV, oxidizing agents, or high levels of growth hormones in the living organisms (viruses and bacteria) stimulates a particular sets of genes such as Hsp to express Hsp to facilitate the stress tolerance and promote cell survival, especially by refolding proteins and preventing their denaturation [9,12]. Moreover it participates in a variety of normal cellular processes including protein trafficking, signal transduction, DNA replication and protein synthesis [13]. The Hsp genes are highly conserved in all eukaryotes and prokaryotes, and based on the sequence homology and molecular weight of proteins; the genes have been divided into families such as Hsp110, Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and Hsp20 [14]. These gene families consist of stress-inducible genes and constitutively expressed genes. Inducible genes are expressed at low levels under non-stress conditions but the level of expression increases in response to stress. In the case of constitutive genes, the basel level expression is high and change relatively little in response to stress [15,16].

In this study, we analyzed the up and down regulation of immune genesb (Lectin (245 bp), PoPO (121 bp), BGBP (166 bp), hemocyanin (242 bp),Toll receptor (150 bp) and Hsp (Hsp21, Hsp70 and Hsp90) genes and categorized them into the small and large heat shock protein family of shrimp *Liptopeneus vannmeai* against WSSV.

Materials and Methods

Preparation of viral inoculum

Viral inoculum was prepared from WSSV-infected shrimp, *Litopenaeus vannamei*, with prominent white spots collected from shrimp farms located near Nellore, India. Haemolymph was drawn directly from the heart of infected shrimp using a sterile syringe. The pooled hemolymph was centrifuged at 3000 g for 20 min at 4°C. The supernatant fluid was recentrifuged at 8000 g for 30 min at 4°C and the final supernatant fluid was filtered through a 0.45 µm filter. The filtrate was then stored at -20°C for infectivity studies. The total protein in haemolymph was determined by Lowery method [17]. The presence of WSSV in the hemolymph was checked by Polymerase Chain Reaction (PCR) using published primers [18]. The healthy *Litopenaeus vannamei* were intramuscularly injected with viral inoculum and shrimp meat prepared from the moribund shrimp was used in WSSV challenge experiment. Shrimp meat from healthy shrimp was prepared and screened by nested PCR for negative control group [18].

Collection and maintenance of experimental animals

Litopenaeus vannamei (8–10 g body weight), were collected from the sea and were maintained in 1000-l fiberglass tanks with air-lift biological filters at room temperature (27-30°C) with salinity between 20 and 25 parts per thousand (ppt). Natural seawater was used in all the experiments. It was pumped from the Bay of Bengal, near Chennai and allowed to sediment to remove the sand and other suspended particles. The seawater was first chlorinated by treating it with sodium hypochlorite at a concentration of 25 parts per million (ppm) and then dechlorinated by vigorous aeration, before being passed through a sand filter and used for the experiments. The animals were fed with artificial pellet feed (CP feed, Thailand). Temperature, pH, salinity and dissolved oxygen were also recorded. From the experimental animals, a small portion of pleopods was excised and used for screening of WSSV by PCR using the primer designed [18].

Experimental challenge and sample collection

The healthy and WSSV negative animals (10/group/tank) were maintained in 100-l aquarium tanks at room temperature (27-30°C) with fresh seawater. The experimental animals were injected intramuscularly using a 1 ml insulin syringe in the third abdominal segment with WSSV inoculum (300 µg of total protein per animal) derived from infected shrimp. Control shrimps were injected with normal saline, for the analysis of expression of immune genes (Lectin (245 bp), PoPO (121 bp), BGBP (166 bp), hemocyanin (242 bp), Toll receptor (150 bp) and heat shock proteins (Hsp90, Hsp70 and Hsp21). Haemolymph samples were randomly collected from experimental animals (3 animals per time point) at 0, 2, 4, 6, 8, 10, 12, 24, 36 and 48 hrs after injection. One milliliter of haemolymph was drawn directly from the heart of experimentally WSSV injected shrimps by inserting a 23 gauge needle attached to a 2 ml syringe containing 1 ml of ice-cold anticoagulant Alsever solution (27 mM Na citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 4.6 at 28°C). The low pH and EDTA in Alsever solution functioned as an anticoagulant, also preventing degranulation and cell lysis in crustacean hemocytes [19]. Target organs (gills, pleopods, hepatopancrease and muscle) were also dissected out from shrimp at 0, 2, 4, 6, 8, 10, 12, 18, 24, 36 and 48 h after WSSV injection separately for RT-PCR. For the analysis of immunological parameters, haemolymph samples were randomly collected from three experimental animals after killing them at 0, 2, 4, 6, 8, 10, 12, 18, 24, 36 and 48 h post-challenge. Haemolymph was also collected from the control animals for the analysis of immunological assays and the results obtained were compared with experimental animals. All the experiments were carried out in triplicate.

RNA extraction and cDNA synthesis

RT-PCR was carried out to study the expression of immune genes ((Lectin, PoPO, BGBP, hemocyanin,Toll receptor) and Hsp genes (ef1α, Hsp90, Hsp70 and Hsp21) of shrimps exposed to WSSV, herbal immune stimulant and normal control. For the isolation of total RNA, the tissues of shrimp after exposure to WSSV were homogenized in RNA isolation reagent Trizol (Gibco BRL, Life Technologies, Rockville, USA), as per the manufacturer's instructions. The extracted RNA was dissolved in nuclease free water. The contaminating DNA was removed by treatment with DNase I (Bangalore Genei, India) at 37°C for 30 min and then re-extracted with phenol–chloroform. RNA integrity was electrophoretically verified by ethidium bromide staining and the purity was checked by measuring the ratio of OD260 nm/ OD280 nm. The DNase-treated total RNA was added with 10 µl DEPC-water containing 100 pmol oligo-dT primers and then denatured by heating at 85°C for 10 min). The first strand cDNA was synthesized by the addition of 3 µl XM-MuLV buffer, 1 µl 100 mM DTT, 1 µl 10 mM dNTPs, 10 U RNasin (Bangalore Genei, India) making a total volume of 10 µl including 100 U M-MuLV reverse transcriptase (New England Biolabs, Beijing, China). The reaction was allowed to proceed at 37°C for 1 h. The cDNA reaction products were subjected to PCR as described below with the primers specific to Hsp genes of shrimps and ef1α. The sequences of primers used in the present study are given in Table 1. The ef1α served as an internal control for RNA quality and amplification efficiency. For PCR, the primers specific to immune genes (Lectin, PoPO, BGBP, hemocyanin, Toll receptor) and Hsp genes of shrimps were used to amplify the PCR product of 100, 123, 145 and 123 bp of Hsp21, Hsp70, Hsp90 and ef1α respectively. Each PCR reaction was in a 25 µl volume containing both forward and reverse primers (10 µM, 0.5 µl each), $MgCl_2$ (25 mM, 1.5 µl) dNTPs (2 mM, 2.0 µl), PCR 10X buffer (12.5 µl), Taq-DNA (1 U, 0.5 µl), template DNA (100 ng) and nucleic acid free water (16.5 µl). PCR consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, annealing temperature of 57°C for 1 min, 72°C for 30 s, and a final extension of 10 min at 72°C. The PCR products were analysed by electrophoresis in 0.8% agarose gels stained with ethidium bromide, and visualized by ultraviolet transillumination.

In vivo transcriptional analyses of immune genes

For immune genes transcriptional analyses, tissue samples of experimentally WSSV injected shrimp, herbal immune stimulant pound treated and uninfected control shrimp were collected at different time intervals.

Symbol	Gene name	Forward primer sequence (5′-3′) Reverse primer sequence (5′-3′)	Gene Bank accession number	Product size (bp)
Hsp21	Heat shock protein 21	AAT TCA TTG CGG AAG CGA GCC A ACT TCA GCG TGA TCG ACC AGG AAT	JF801919	100
Hsp70	Heat shock protein 70	AGA AGT CAC TCC GTG ATG CCA AGA ACT CCT TGC CGT TGA AGA AGT CCT	AF474375	123
sp90	Heat shock protein 90	GCA TGA AGG AGA ACC AGA AGC ACA TGA ACG CAG TAT TCG TCG ATG GGT	FJ855436	145
ef1α	Internal control gene	GGC GTA CTG GTA AGG AAC TGG AA GAG GAG CAT ACT GTT GGA AGG TCT C	AB458256.1	123
Toll	Toll receptor	CTTCCCTCCTGCTCTGCT ACCACTCAGGCAACAGGG	EF117252	143
PoPo	Prophenol oxidase	TCTGGGTCTCCCGAAGGT AACCGTCGCACAACAGGA	AF099741.1	128
Hemecyanin	Hemocyanin	TTGCGGTCATCCACTCAC AACAGCAGGGTGGTCTTA	JF357966	262
Lectin	Lectin 214	ACTGGTGCCCGAAAGAAA ACGGGTGACACTTCCAATAA	DQ871244	214
BGBP	BGBP	AATGATTTCTATCCCACTC AGTGAAGCCAAGGTGAAT	AY249858.1	153

Table 1: GenBank accession numbers, gene name, and primer sequences of *Hsp* genes used for real-time RT-PCR analysis.

One group of samples was subjected to RT-PCR analysis using the specific primers for the immune genes (Lectin, PoPO, BGBP, hemocyanin, Toll receptor) gene primers (Table 1) to determine the expression of immune genes (Lectin, PoPO, BGBP, hemocyanin,Toll receptor (150 bp)) and for transcriptional analysis. The housekeeping gene ef1α, was used as an internal control for all RT-PCR experiments (primers ef1α F+R, 123 bp; Table 1). The samples were subjected to RT-PCR and the detailed procedure is described above.

Tissue distribution and *In vivo* transcriptional analyses of Hsps

For Hsps distribution and transcriptional analyses, tissue samples (gill, hepatopancreas, pleopods and muscle) of experimentally WSSV injected shrimp and uninfected control shrimp were collected at different time intervals 0, 2, 4, 6, 8, 10, 12, 18, 24, 36 and 48 h post-treatment. One group of samples was subjected to RT-PCR analysis using the specific primers for the Hsps (Hsp90, Hsp70 and Hsp 21) gene primers (Table 1) to determine the expression of Hsps (Hsp90, Hsp70 and Hsp21) and for transcriptional analysis. The housekeeping gene ef1α, was used as an internal control for all RT-PCR experiments (primers ef1α F + R, 123 bp; Table 1). The samples were subjected to RT-PCR and the detailed procedure is described above.

Quantification of Hsp21, Hsp70 and Hsp90 mRNA expression by real-time qRT-PCR

The abundance of the ef1α, Hsp21, Hsp70 and Hsp90 transcripts was measured by real-time quantitative reverse transcription PCR (real-time qRT-PCR) to determine the CT (cycle threshold) value of WSSV infected shrimp and normal shrimp at different time intervals (p.i.) [20]. Total RNA extracted from WSSV infected shrimp and normal shrimp tissue samples were collected at different time intervals and cDNA was converted by the method described above. The cDNA was quantified using the NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, USA) at 260 nm. The primers specific to ef1α, Hsp21, Hsp70 and Hsp90 were used (Table 1) to amplify a 123, 100, 123 and 145 bp fragment, respectively. The ef1α, Hsp21, Hsp70 and Hsp90 load was estimated by StepOnePlusTM system (Applied Biosystems, Singapore) using DyNAmoTMSYBR Green qPCR Kit (Finnzymes, Espoo, Finland). Reaction mixtures of 10 µl were analyzed in triplicate. Each PCR plate had a negative control in which cDNA from uninfected shrimp was added as template. The cycling parameters used were as follows; an initial denaturation at 95°C for 3 min, 40 cycles denaturation at 95°C for 20 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s. The house keeping gene, ef1α was used as an internal control for all real time PCR experiments.

Immunological assay

The immunological assays such as prophenoloxidase assay (proPO), superoxide anion assay, superoxide dismutase activity and nitric oxide assay were analyzed for different groups. Prophenoloxidase activity was spectrophotometrically recorded by measuring the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) according to Ashida and Soderhall [21]. Superoxide anion assay was quantified as described by Song and Hsieh [22]. Superoxide dismutase activity was determined according to Beauchamp and Fridovich [23]

using Nitroblue Tetrazolium (NBT) in the presence of riboflavin. Spectrophotometric determination of haemolymph total nitrite and nitrate levels was carried out following the procedure of Sastry et al. [24]. To determine the total nitrite and nitrate the acidic Griess reaction method for color development after the reduction of nitrate with copper cadmium alloy and deproteinization was used.

Enzyme-Linked Immunosorbent Assay (ELISA)

The expression of heat shock protein gene (Hsp21, Hsp70 and Hsp90) in four different tissues (muscle, hepatopancreas, gill and pleopod) of WSSV injected *L. vannamei* at 0, 2, 4, 6, 8, 10, 12, 18, 24, 36 and 48 h was also confirmed by ELISA using the commercially available antiserum raised against heat shock protein gene (Hsp 21, Hsp 70 and Hsp 90). The wells of flat-bottomed ELISA plate were coated with the suspension of tissue (muscle, hepatopancreas, gill and pleopod) samples of WSSV injected *L. vannamei* in PBS overnight at 4°C. The plates were then washed thoroughly with PBS and blocked with 1% BSA in PBS for 1 h at 37°C. Subsequently, the plates were washed thoroughly with PBS/T and incubated with antiserum raised against Hsp (21, 70 and 90) at 37°C for 2 h. The plates were washed with PBS/T and PBS three times each for 2 min and further incubated with 100 µl of rabbit anti-mouse IgG conjugated with alkaline phosphatase for 1 h. The plates were washed with PBS/T and PBS three times each for 2 min and developed with the substrate p-nitrophenyl phosphate in substrate buffer. The optical density was measured at 405 nm using an automated ELISA reader (Labsystems, USA).

Statistical Analysis

Data are expressed as mean ± SE. The Mann Whitney non-parametric tests and parametric student's t test were used for tests of significance of differences between groups. A probability level of 0.05 was used to assess significance in all measured parameters. Statistical calculations were performed using SPSS (Version9) software.

Results

RT-PCR analysis of heat shock proteins after WSSV injection at room temperature

Transcriptional analysis of heat shock proteins (Hsp21, 70 and 90) showed that in WSSV injected shrimps, Hsp21 was down regulated in all experimental organs (Figure 1), whereas Hsp70 was up regulated in all experimental organs with different time course after WSSV injection (Figure 2). The Hsp90 also was down regulated in all experimental organs with different time course after injection of WSSV (Figure 3). All the test results were compared with internal control eflα (Figure 4).

Tissue expression pattern of the Hsps time course induction of the Hsp genes in different organs in WSSV infected shrimps (*L. vannamei*)

To assess a time-dependent induction of the Hsp gene expression, Cycle Threshold (CT) values of the Hsp21, Hsp70, Hsp90 and ef1α transcripts were measured in L. vannamei exposed to the WSSV infection by real-time PCR (Tables 2-5). The result showed that Hsp21 and Hsp90 were down regulated in almost all organs such as muscle, hepatopancreas, gill and pleopod when compared with normal shrimp (Tables 3 and 5). The Hsp21 and Hsp90 gene expression decreases during the course of infection in the *Litopenaeus vannamei* infected with WSSV and a lower CT value of Hsp21 and Hsp90 were observed at 0 hours p.i. whereas a higher CT value was observed at 48 hours p.i. The Hsp70 gene expression increase during the course of infection in the *Litopenaeus vannamei* infected with WSSV, higher CT value was observed at 0 hours p.i. whereas a lower CT value was observed at 48 hours p.i. (Table 4). No significant difference was observed in the CT value of ef1α in the *Litopenaeus vannamei* infected with WSSV during the time course of infection (Table 2). The Coefficient of Variation (CV) for the CT values of Hsp21, Hsp70 Hsp90 and ef1α gene was found to be less than 5% and this indicated that the assay was highly reproducible (Tables 2-5).

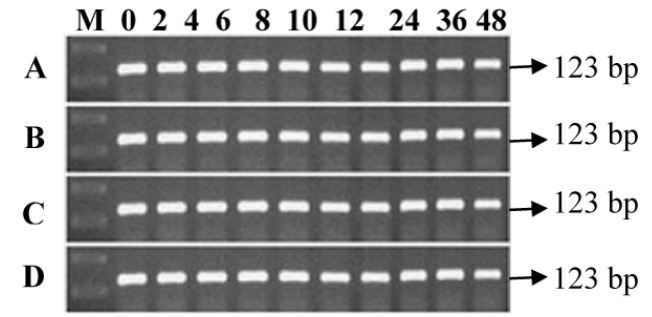

Figure 1: RT-PCR time course analysis of *ef1α* (123 bp, used as an internal control) expression in different tissues of white shrimp *Litopenaeus vannamei* at 0, 2, 4, 6, 8, 10, 12, 24, 36 and 48 h post injection of WSSV. Lane M- marker; Land 2 to 12 as 0, 2, 4, 6, 8, 10, 12, 24, 36 and 48 h. A. *ef1α* expression in gill tissue B. *ef1α* expression in muscle tissue C. *ef1α* expression in hepatopancrease D. *ef1α* expression in pleopod.

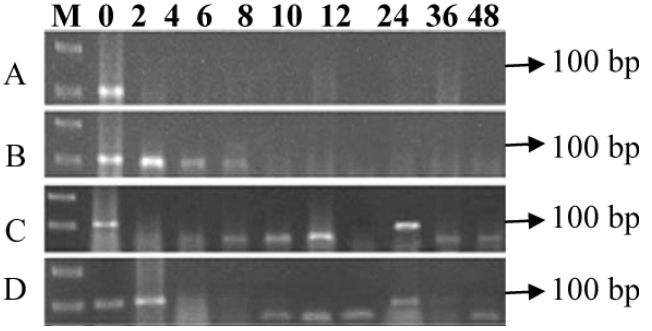

Figure 2: RT-PCR time course analysis of Hsp 21 (100 bp) expression in different tissues of white shrimp *Litopenaeus vannamei* at 0, 2, 4, 6, 8, 10, 12, 24, 36 and 48 h post injection of WSSV. Lane M- marker; Land 2 to 12 as 0, 2, 4, 6, 8, 10, 12, 24, 36 and 48 h. A. Hsp 21 expression in gill tissue B. Hsp 21 expression in muscle tissue C. Hsp 21 expression in hepatopancrease and D. Hsp 21 expression in pleopod.

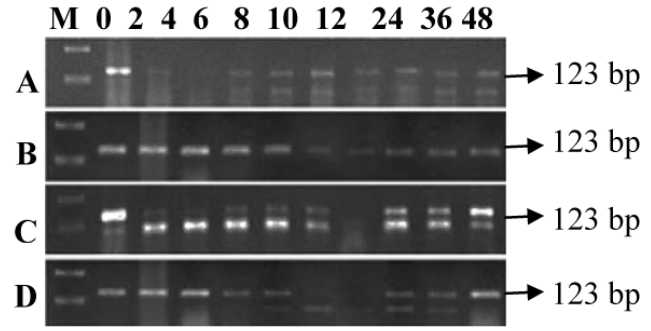

Figure 3: RT-PCR time course analysis of Hsp70 (123 bp) expression in different tissues of white shrimp *Litopenaeus vannamei* at 0, 2, 4, 6, 8, 10, 12, 24, 36 and 48 h post injection of WSSV. Lane M- marker; Land 2 to 12 as 0, 2, 4, 6, 8, 10, 12, 24, 36 and 48 h. A. Hsp70 expression in gill tissue B. Hsp70 expression in muscle tissue C Hsp70 expression in hepatopancrease D. Hsp70 expression in pleopod.

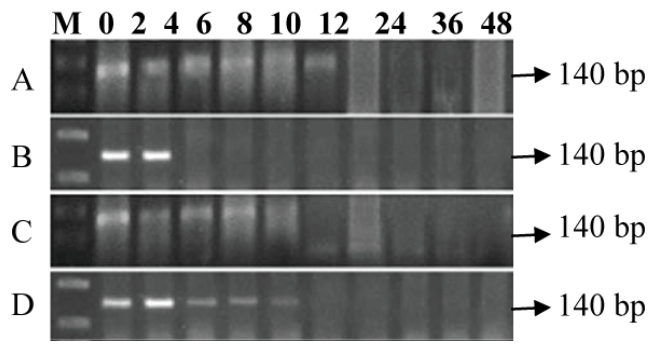

Figure 4: RT-PCR time course analysis of Hsp 90 (140 bp) expression in different tissues of white shrimp *Litopenaeus vannamei* at 0,2,4,6,8,10,12,24,36 and 48 h post injection of WSSV. Lane M- marker; Land 2 to 12 as 0,2,4,6,8,10,12,24,36 and 48 h. A. Hsp 90 expression in gill tissue B. Hsp 90 expression in muscle tissue C. Hsp 90 expression in hepatopancrease D. Hsp 90 expression in pleopod.

Hours	elfα gene CT/µl of total extracted RNA at different time of post infection in *Penaeus monodon*															
	Muscle				Hepatopancreas				Gills				Pleopod			
	CT	CT Mean	SD	CV	CT	CT Mean	SD	CV	CT	CT Mean	SD	CV	CT	CT Mean	SD	CV
0	24.54 24.22 24.54	24.43	0.18	0.73	25.13 25.41 25.84	24.43	0.18	0.73	24.15 24.84 24.54	24.51	0.34	1.38	21.54 21.34 21.26	21.38	0.14	0.65
2	24.25 24.84 24.04	24.37	0.41	1.68	24.15 24.56 24.87	24.37	0.41	1.68	25.47 25.77 25.34	25.52	0.22	0.86	21.86 21.13 21.44	21.47	0.36	1.67
4	25.33 25.85 25.13	25.43	0.37	1.45	24.03 24.64 24.34	25.43	0.37	1.45	24.57 24.61 24.37	24.51	0.12	0.48	21.85 21.14 21.34	21.44	0.36	1.67
6	25.93 25.14 25.31	25.46	0.41	1.61	24.16 24.86 24.46	25.46	0.41	1.61	26.48 26.75 26.07	26.43	0.34	1.28	22.64 22.44 22.74	22.60	0.15	0.66
8	24.34 24.67 24.54	24.53	0.16	0.65	25.61 25.34 25.75	24.51	0.16	0.65	24.62 24.84 24.41	24.62	0.21	0.85	22.46 22.14 22.87	22.49	0.36	1.60
10	24.61 24.84 24.14	24.51	0.35	1.42	24.61 24.33 24.84	24.53	0.35	1.42	25.34 25.84 25.41	25.53	0.27	1.05	21.84 21.43 21.49	21.58	0.22	1.01
12	25.33 25.47 25.16	24.53	0.15	0.61	25.76 25.34 25.47	25.32	0.15	0.59	24.74 24.84 24.31	24.63	0.28	1.13	21.76 21.16 21.46	21.46	0.30	1.39
24	24.56 24.88	24.53	0.36	1.46	24.65 24.04	24.53	0.36	1.46	26.48 26.31	26.54	0.27	1.01	22.64 22.37	22.60	0.22	0.97

	24.16				24.44				26.84				22.81			
36	25.34 25.41 25.84	25.53	0.27	1.05	25.41 25.86 25.64	25.53	0.27	1.05	26.45 26.34 26.84	26.54	0.26	0.97	21.44 21.61 21.86	21.63	0.21	0.97
48	24.31 24.84 24.61	24.58	0.26	1.05	25.27 25.44 25.16	25.29	0.14	0.55	24.34 24.65 24.24	24.41	0.21	0.86	22.47 22.34 22.19	22.33	0.14	0.62
Negative	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 2: Cycle Threshold (C_T) Mean values of total RNA in different organs of *Lipotopeneusvannamei* infected with WSSV at different time intervals.. C_T: Cycle Threshold; SD: Standard Deviation; CV: Coefficient Of Variation; ND: Not Determined

Hours	HSP21 gene CT/µl of total extracted RNA at different time of post infection in *Penaeus monodon*															
	Muscle				Hepatopancreas				Gills				Pleopod			
	C_T	C_T Mean	SD	CV	C_T	C_T Mean	SD	CV	C_T	C_T Mean	SD	CV	C_T	C_T Mean	SD	CV
0	27.51 27.94 27.84	27.76	0.22	0.79	25.69 25.80 25.34	25.61	0.24	0.93	27.57 27.24 27.88	27.56	0.32	1.16	22.92 22.34 22.51	22.59	0.29	1.28
2	28.88 28.17 28.34	28.46	0.37	1.30	26.34 26.84 26.04	26.40	0.40	1.51	28.77 28.39 28.07	28.41	0.35	1.23	24.87 24.66 24.79	24.77	0.10	0.40
4	30.14 30.84 30.55	30.51	0.35	1.14	28.44 28.37 28.89	28.56	0.28	0.98	30.64 30.57 30.91	30.70	0.17	0.55	25.64 25.34 25.94	25.64	0.30	1.17
6	31.64 31.87 31.99	31.83	0.17	0.53	28.34 28.04 28.56	28.31	0.26	0.91	31.54 31.35 31.47	31.45	0.09	0.28	26.34 26.84 26.54	26.57	0.25	0.94
8	31.24 31.07 31.34	31.21	0.13	0.41	29.64 29.37 29.19	29.40	0.22	0.74	31.87 31.15 31.04	31.35	0.45	1.43	27.35 27.34 27.27	27.32	0.04	0.14
10	32.84 32.66 32.17	32.55	0.34	1.04	30.54 30.88 30.31	30.57	0.28	0.91	32.63 32.84 32.17	32.54	0.34	1.04	28.64 28.39 28.44	28.49	0.13	0.45
12	33.76 33.59 33.84	33.73	0.12	0.35	32.76 32.46 32.19	32.47	0.28	0.86	32.04 32.54 32.19	32.25	0.25	0.77	28.66 28.37 28.04	28.35	0.31	1.09
24	33.04 33.46 33.19	33.23	0.21	0.63	33.48 33.94 33.83	33.75	0.24	0.11	33.94 33.24 33.84	33.67	0.37	1.09	30.18 30.64 30.94	30.58	0.38	1.24
36	33.09 33.37 34.76	33.74	0.89	2.63	33.04 33.34 33.29	33.22	0.16	0.48	33.24 33.61 33.04	33.29	0.28	0.84	32.14 32.85 32.37	32.45	0.36	1.10
48	34.67 34.04	34.33	0.31	0.90	34.56 34.80	34.48	0.36	1.04	34.86 34.49	34.57	0.25	0.72	33.76 33.31	33.67	0.32	0.95

	34.29				34.09				34.37				33.94			
Negative	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 3: Cycle Threshold (C_T) Mean values of total RNA in different organs of Liptop*enaeus vannamei* infected with WSSV. C_T: Cycle Threshold; SD: Standard Deviation; CV: Coefficient of Variation; ND: Not Determined.

Hours	HSP70 gene CT/µl of total extracted RNA at different time of post infection in *Penaeus monodon*															
	Muscle				Hepatopancreas				Gills				Pleopod			
	CT	CT Mean	SD	CV	CT	CT Mean	SD	CV	CT	CT Mean	SD	CV	CT	CT Mean	SD	CV
0	29.84 29.99 29.73	29.85	0.13	0.43	29.87 29.79 29.63	29.76	0.12	0.40	30.84 30.43 30.59	30.62	0.20	0.65	33.54 33.77 33.69	33.66	0.11	0.32
2	29.54 29.34 29.30	29.39	0.12	0.40	29.44 29.13 29.07	29.21	0.19	0.65	30.14 30.08 30.54	30.25	0.25	0.82	33.17 33.29 33.34	33.26	0.08	0.24
4	29.34 29.27 29.53	29.38	0.13	0.44	28.94 29.02 28.73	28.89	0.14	0.48	29.87 29.73 29.54	29.71	0.16	0.53	32.56 32.37 32.64	32.52	0.13	0.39
6	28.66 28.81 28.73	28.73	0.07	0.24	28.53 28.47 28.64	28.54	0.08	0.28	29.44 29.31 29.19	29.31	0.12	0.40	32.24 32.32 32.18	32.24	0.07	0.21
8	28.34 28.14 28.22	28.23	0.10	0.35	28.34 28.11 27.97	28.14	0.18	0.63	28.44 28.79 28.87	28.70	0.22	0.76	31.99 31.91 31.84	31.91	0.07	0.21
10	27.94 27.44 27.67	27.68	0.25	0.90	27.94 27.81 27.99	27.91	0.09	0.32	28.34 28.46 28.57	28.45	0.11	0.38	31.54 31.67 31.41	31.54	0.13	0.41
12	27.64 27.49 27.31	27.48	0.16	0.58	27.44 27.53 27.76	27.57	0.16	0.58	27.64 27.45 27.37	27.48	0.13	0.47	30.41 30.74 30.61	30.58	0.16	0.52
24	27.16 27.39 27.21	27.25	0.12	0.44	27.16 27.04 27.29	27.16	0.12	0.44	26.54 26.84 26.73	26.70	0.15	0.56	29.34 29.59 29.27	29.40	0.16	0.54
36	26.94 26.43 26.82	26.73	0.26	0.97	26.88 26.71 26.97	26.85	0.13	0.48	25.49 25.84 25.88	25.73	0.21	0.81	28.74 28.44 28.37	28.51	0.19	0.66
48	26.54 26.29 26.37	26.40	0.12	0.45	26.44 26.39 26.59	26.47	0.10	0.37	25.27 25.18 25.06	25.17	0.10	0.39	28.28 28.06 28.34	28.22	0.14	0.49
Negative	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 4: Cycle Threshold (C_T) Mean values of total RNA in different organs of *Litopenaeus vannamei* infected with WSSV. C_T: Cycle Threshold; SD: Standard Deviation; CV: Coefficient of Variation; ND: Not Determined.

Hours	HSP90 gene CT/µl of total extracted RNA at different time of post infection in *Penaeus monodon*															
	Muscle				Hepatopancreas				Gills				Pleopod			
	CT	CT Mean	SD	CV	CT	CT Mean	SD	CV	CT	CT Mean	SD	CV	CT	CT Mean	SD	CV
0	26.71 26.53 26.46	26.56	0.12	0.45	30.43 30.87 30.67	30.65	0.22	0.71	25.50 25.84 25.64	25.66	0.17	0.66	26.48 26.94 26.87	26.76	0.24	0.89
2	27.44 27.84 27.94	27.74	0.26	0.93	31.44 31.24 31.87	31.51	0.32	1.01	26.48 26.76 26.38	26.54	0.19	0.71	27.65 27.34 27.54	27.51	0.15	0.54
4	27.32 27.13 27.40	27.28	0.13	0.47	32.98 32.76 32.57	32.77	0.20	0.61	26.04 26.22 26.13	26.13	0.09	0.34	28.44 28.34 28.67	28.48	0.16	0.56
6	28.79 28.81 28.64	28.74	0.09	0.31	32.26 32.44 32.13	32.27	0.15	0.46	27.83 27.64 27.49	27.65	0.17	0.61	29.64 29.55 29.84	29.67	0.14	0.47
8	28.44 28.64 28.35	28.47	0.14	0.49	33.87 33.66 33.94	33.82	0.14	0.41	27.35 27.12 27.08	27.18	0.14	0.51	32.41 32.84 32.56	32.60	0.21	0.65
10	29.64 29.54 29.87	29.68	0.16	0.53	33.23 33.61 33.42	33.42	0.19	0.56	28.74 28.64 28.44	28.60	0.15	0.52	32.14 32.06 32.34	32.18	0.14	0.43
12	29.31 29.16 29.27	29.24	0.07	0.23	34.88 34.56 34.75	34.73	0.16	0.46	28.24 28.06 28.11	28.13	0.09	0.31	32.01 32.14 33.84	32.66	1.02	3.12
24	30.44 30.87 30.94	30.75	0.27	0.87	35.37 35.84 35.46	35.55	0.24	0.67	29.44 29.79 29.62	29.61	0.17	0.57	33.91 33.84 33.67	33.80	0.12	0.35
36	30.82 31.24 31.37	31.14	0.28	0.89	36.11 35.76 36.04	35.97	0.18	0.50	29.34 29.17 29.29	29.26	0.08	0.27	33.24 33.39 33.12	33.25	0.13	0.39
48	31.56 31.33 31.48	31.45	0.11	0.34	36.49 36.87 36.67	36.67	0.19	0.51	30.31 30.47 30.64	30.47	0.16	0.52	34.93 34.67 34.27	34.62	0.33	0.95
Negative	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 5: Cycle Threshold (C_T) Mean values of total RNA in different organs of *Litopenaeus vannamei* infected with WSSV. C_T: Cycle Threshold; SD: Standard Deviation; CV: Coefficient of Variation; ND: Not Determined.

Immune parameters

Prophenoloxidase levels among the two groups of shrimps showed a significant increase when compared with the control group. In WSSV infected shrimp, proPO levels were significantly increased and reached double the normal value at moribund state (Figure 5A). In WSSV-infected shrimp, superoxide dismutase activity decreased and reached lowest level at moribund stage in comparison with the control group (Figure 5B). Shrimp infected with WSSV by intramuscular injection showed a significant increase in superoxide anion concentration in comparison with the control group (Figure 5C). Shrimp infected with WSSV by intramuscular injection showed a significant increase in nitrate and nitrite levels in comparison with the control group (Figures 5D and 5E).

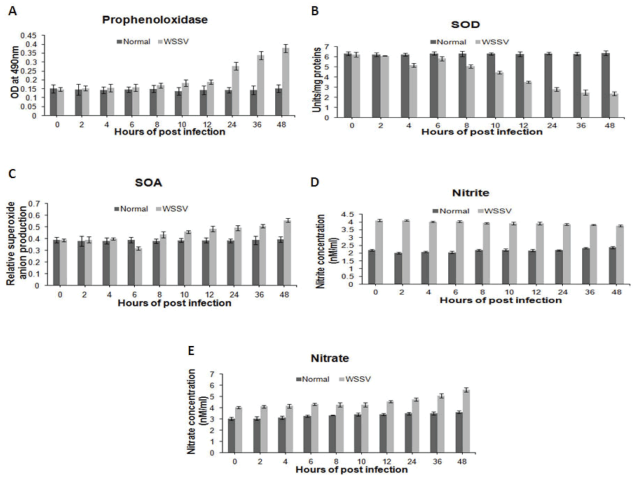

Figure 5: ELISA of HSP expression in different tissues of white shrimp *Litopenaeus vannamei* at 0,2,4,6,8,10,12,24,36 and 48 h post injection of WSSV. A. Hsp 21 B. Hsp 70 C. Hsp 90.

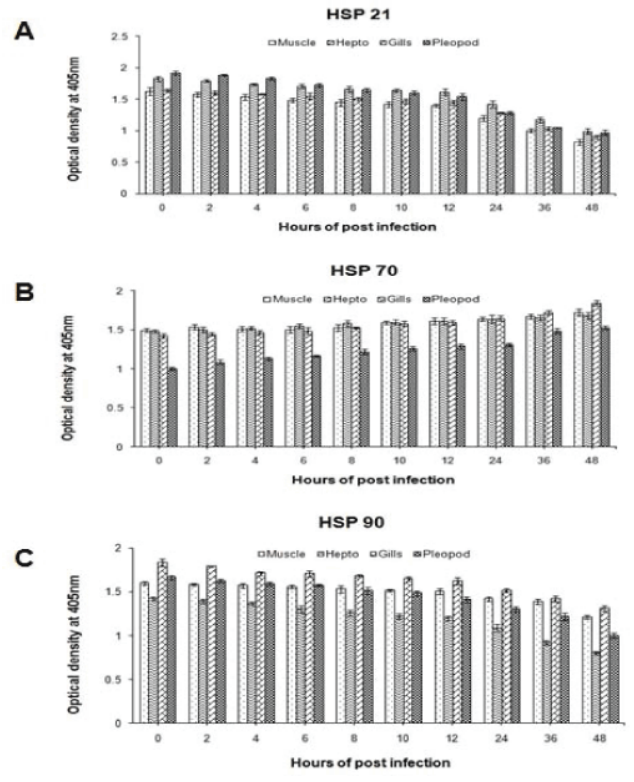

Figure 6: Immunological changes of white shrimp *Litopenaeus vannamei* injected with PBS and WSSV at different time intervals (0, 2, 4, 6, 8, 10, 12, 24, 36 and 48 h) of post infection. A. Prophenoloxidase activity B. Superoxide dismutase activity C. Superoxide anion activity D. Nitrate activity E. Nitrite activity (OD490 nm).

Expression of heat shock protein by ELISA

The expression of Hsp21, Hsp70 and Hsp90 protein could be detected by ELISA (Figure 6) in different tissues (muscle, hepatopancreas, gill and pleopod) of WSSV injected *Litopenaeus vannamei* with different time intervals (0, 2, 4, 6, 8, 10, 12, 18, 24, 36 and 48 h).

RT-PCR analysis of immune genes after WSSV and herbal immune stimulant injection at room temperature

Transcriptional analysis of immune genes (Lectin (245 bp), PoPO (121 bp), BGBP (166 bp), hemocyanin (242 bp), Toll receptor (150 bp)) showed that in WSSV injected shrimps, all the immune genes were down regulated in all experimental animals (Figure 7), whereas all immune genes were was up regulated in all experimental animals with different time course after injection of herbal immune stimulant with WSSV, herbal immune stimulant alone compared with normal control animals (Figures 8-10). All the test results were compared with internal control eflα (Figure 4).

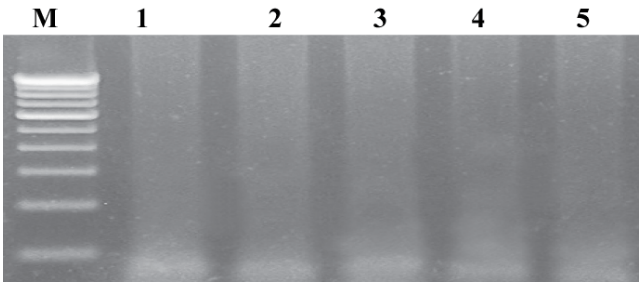

Figure 7: RT-PCR analysis of immune genes (Lectin (245 bp), PoPO (121 bp), BGBP (166 bp), hemocyanin (242 bp),Toll receptor (150 bp)) expression in white shrimp *Litopenaeus vannamei* under exposure of WSSV. Lane M – 100 bp marker; Land 1- Lectin (245 bp), 2 - PoPO (121 bp), 3 - BGBP (166 bp), 4 - Hemocyanin (242 bp), 5 - Toll receptor (150 bp).

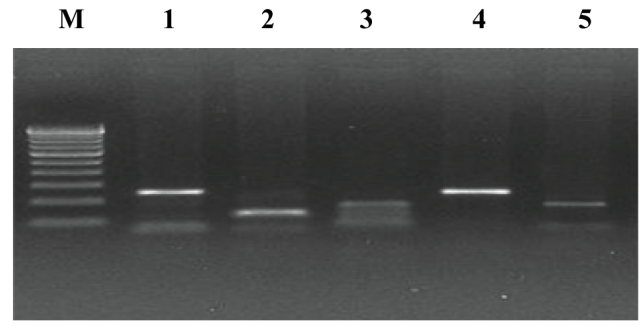

Figure 8: RT-PCR analysis of immune genes (Lectin (245 bp), PoPO (121 bp), BGBP (166 bp), hemocyanin (242 bp),Toll receptor (150 bp)) expression in white shrimp *Litopenaeus vannamei* under exposure of NTE buffer. Lane M – 100 bp marker; Land 1- Lectin (245 bp), 2 - PoPO (121 bp), 3 - BGBP (166 bp), 4 - Hemocyanin (242 bp), 5 - Toll receptor (150 bp).

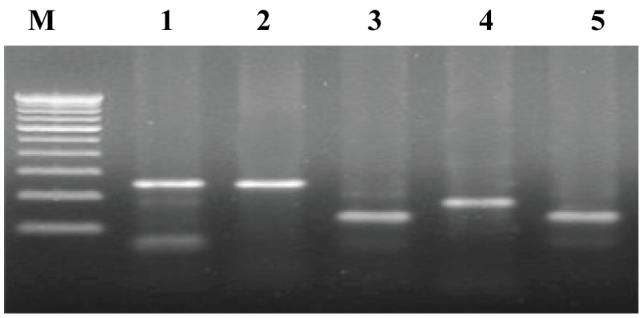

Figure 9: RT-PCR analysis of immune genes (Lectin -245 bp, Hemocyanin -242 bp , PoPO -121 bp, BGBP -166 bp, Toll receptor -150 bp) expression in white shrimp *Litopenaeus vannamei* at 48 h post injection of WSSV+immuzone. Lane M- 100 bp marker; Land 1- Lectin (245 bp), 2- hemocyanin (242 bp) , 3 - PoPO (121 bp), 4 - BGBP (166 bp), 5 - Toll receptor (150 bp).

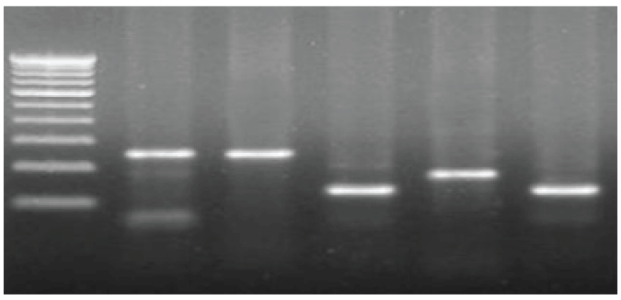

Figure 10: RT-PCR analysis of immune genes (Lectin -245 bp, Hemocyanin -242 bp , PoPO -121 bp, BGBP -166 bp, Toll receptor -150 bp) expression in white shrimp *Litopenaeus vannamei* under exposure of immuzone only. Lane M- 100 bp marker; Land 1- Lectin (245 bp), 2- hemocyanin (242 bp) , 3 - PoPO (121 bp), 4 - BGBP (166 bp), 5 - Toll receptor (150 bp).

Discussion

Diseases caused by viruses are the greatest challenge to worldwide shrimp aquaculture. However, till date, there is no effective method to control viruses, especially White Spot Syndrome Virus (WSSV). Better understanding of shrimp immune responses will be greatly helpful to control the diseases. Heat Shock Proteins (HSPs) are molecular chaperones which are involved in maintaining regular cellular functions with a crucial role in protein folding, unfolding, aggregation, degradation, and transport [25].

Heat shock proteins are known to play a protective role as molecular chaperones to assist in protein folding, protein assembly and damaged proteins degradation [26]. In addition, these highly conserved proteins are involved in cell differentiation and morphogenesis [27], cell signaling, and in the protection of cells against stress and apoptosis [28-30]. While most of the Hsp genes are well characterized in model organisms such as *Drosophila melanogaster* [31-33] very little information is available in the case of *Litopenaeus vannamei*. Although several families of the Hsp genes have been identified, only full length cDNAs of Hsp21, Hsp70 and Hsp90 were characterized in *Litopenaeus vannamei* [34-36]. The extensive studies of the three major families of the heat shock proteins in model organisms demonstrate that Hsp70 has a primary role in assisting folding of nascent polypeptide chains and facilitating in repairing or degradation of damaged proteins [9]. The Hsp90 has been shown to be involved in various cell components such as protein kinases in signal transduction pathways, transcriptional factors, cell cycle regulators, and steroid hormone receptors [37-40]. The Hsp21 acts, as a chaperone by binding to an unfolded protein and mediating in protein folding [40,41]. To gain more insight on the Hsp genes in *Litopenaeus vannamei*, the expression profiles of Hsp21, Hsp70 and Hsp90 in different tissues and the time-course induction patterns of these genes under WSSV treated in gill, muscle, pelopad and hepatopancreas of *Litopenaeus vannamei* were assessed.

The expression and distribution of patterns of Hsp21, Hsp70 and Hsp90 were quantified in four different tissues of Litopenaeus vannamei. Under WSSV treated condition, all the three Hsp genes were commonly expressed in all exmained tissue suggesting that all three forms of Hsp gene products were required to maintain cellular homeostasis. However, their expression levels varied in each tissue. The Hsp21 transcript was the most abundant in gill whereas the transcript of Hsp70 was mostly expressed in hepatopancreas and Hsp90 showed no significant difference in its basal expression level. The differential expression levels in different organs are observed in other model organisms. In flesh fly (*Sarcophaga crassipalpis*), the expression levels of Hsp21, Hsp70, and Hsp90 are highest in brain, followed by epidermis and mid-gut. Among the three genes, the Hsp70 transcript was expressed at a higher level suggesting that Hsp70 was a dominant form of heat shock response proteins. On the other hand, the Hsp21 and Hsp90 levels were less abundant in comparison to that of the Hsp70. The more abundance of the Hsp70 than Hsp21 and Hsp90 from our results was also consistent with the higher frequency of Hsp70 clones found in the *L. vannamei* EST libraries than the Hsp21 or Hsp90 [42]. In addition, our results were consistent with a previous report that the *L. vannamei* Hsp70 is the heat shock cognate isoform. Therefore, Hsp70 in *P. monodon* is constitutively and highly expressed acting as a housekeeping gene whose protein plays aputative role as defense mechanism against cell damages [43]. To further investigate the involvement of the Hsp genes in immune response, the expression profiles of Hsp21, Hsp70 and Hsp90 in gill of *L. vannamei* exposed with WSSV were determined. Upon pathogen invasion, *L. vannamei* triggers innate immune responses as their defense mechanisms and the well-known mechanism of shrimp innate immune system against microbial infection is phagocytosis and dominantly takes place in hemocytes [44].

The phagocytosis process involves the release of Reactive Oxygen Species (ROS) to kill pathogenic bacteria. Since HSPs have been well characterized as molecular chaperones, which assist in protein folding and repairing along with the significant induction of the Hsp70 and Hsp90 transcripts in hemocytes under heat shock stress it also suggests the possible involvement of Hsp in shrimp immune response. This result provides the evidence for putative roles of protein products of Hsp21, Hsp70 and Hsp90 in an immune response, especially in the case of the highly induced Hsp90 expression level during the WSSV challenge. In mammalian systems, heat shock proteins have been proposed to act as signaling molecules to activate the host innate immune response. Heat shock proteins such as Hsp70 and Hsp90 are

released from damaged cells to interact with host immune cells [45,46]. Although the stimulation of innate immune responses by heat shock proteins in hosts has not yet been extensively characterized in invertebrates, many reports have shown the correlation between the increased levels of heat shock proteins and the reduction of pathogens such as the induction of Hsp70 by a short exposure to heat shock which results in the reduction of Gill-Associated Virus (GAV) in infected *P. monodon* [47]. Additionally, we hypothesize that the viral infections such as WSSV may cause similar tissue and protein damages to heat shock stress. Therefore, upon the presence of pathogens, shrimp induced the expression of the Hsp genes, which may act as chaperone proteins to repair protein damages and may play additional role as signaling molecules to modulate innate immune response in host shrimp.

The results agree our studies revealed that Hsp21 and Hsp 90 showed lower level of expression when compared to Hsp70. Among the three Hsp genes, the Hsp90 of *L. vannamei* showed a distinct, consistent and rapidly responsive expression pattern under the WSSV challenge. This work provides the evidence to support the putative roles of Hsp21, Hsp70 and Hsp90 as a part of the host immune response. Further studies on the involvement of heat shock proteins under environmental and pathogenic stresses will be essential to elucidate the protective roles of heat shock proteins in host shrimp.

Phenoloxidase (PO) is the terminal enzyme in the proPO system of the arthropod defense system and acts as both recognition and effector component, by promoting cell-to-cell communication and subsequently eliminating pathogens [48,49]. The active materials formed during the activation of proPO stimulate several cellular defense reactions, including phagocytosis, nodule formation, hemocyte locomotion, non-self-recognition and other immune reactions [50]. Activated phenoloxidases generate high cytotoxic quinines that can inactivate viral pathogens [51].

WSSV-infected shrimp groups showed elevated levels of proPO levels, 48 h.p.i. group showed increased levels of proPO which was almost double the level seen in healthy shrimp. Zhang et al. reported that the PO in the hemocytes of WSSV infected Fenneropenaeus chinensis increased significantly, which is consistent with the present results [52]. The proPO activating mechanism of WSSV is still unknown. Le Moullac et al. reported that a high level of PO activity sometimes arises as an inefficient compensation mechanism to maintain resistance against infection [53]. This might be the reason for increase of PO in WSSV infection. The active materials formed during the activation of proPO stimulate several cellular defense reactions, including phagocytosis, nodule formation, hemocyte locomotion, non-self-recognition and other immune reactions [50].

In the present study, *L. vannamei* infected with WSSV showed increased levels of superoxide anion and decreased levels of SOD after 12 h.p.i. when compared with the healthy shrimp. Liu and Chen [54] eased the level of SOD in *L. vannamei* as observed. This fact suggested that the activity of NADPH oxidase responsible for the release of superoxide anion increased together with a decrease in the activity of superoxide dismutase, which was responsible for scavenging superoxide anion. Singlet oxygen and hydroxyl radicals were reported to inactivate SOD with resultant loss of enzyme activity [55]. A small increase in the superoxide anion is considered to be beneficial with respect to enhancement of immunity [56]. However, too great an increase of superoxide anion may be toxic to the host [57].

The results obtained from the ELISA showed a significant increase in the Hsp immuno-reactivity in WSSV infected animal. The development of the ELISA in this investigation is believed to be the first assay to quantitatively measure an Hsp in crustaceans. Using ELISA, it is now possible to test a large number of samples quickly, easily and at relatively low cost. The development of this assay provides a means to screen farmed and wild populations for changes in Hsp levels and may serve as a basis for possible management strategies in times of stress.

In conclusion, this work examined the expression patterns of Hsp21, Hsp70, and Hsp90 under in four tissues of *L. vannamei* infected with WSSV, and Hsp 70 was the most dominant form. The time course induction patterns of the three Hsp transcripts were determined in tissues of *L. vannamei* where Hsp 70 was significantly up-regulated under WSSV infection and Hsp21 and Hsp90 were down-regulated when *L. vannamei* exposed to the presence of WSSV. This work provided the evidence to support the putative roles of Hsp21, Hsp70 and Hsp90 as a part of the host immune response.

Acknowledgement

The first author is recipient of a Young Scientist award from the Department of Science and Technology, Government of India and partially funded by Department Biotechnology (DBT), Government of India. The authors are thankful to the Management of C. Abdul Hakeem College, Melvisharam for providing the facilities to carry out this work.

References

1. Le Moullac G, Le Groumellec M, Ansquer D, Froissard S, Levy P, et al. (1997) Aquacop. Haematological and phenoloxidase activity changes in the shrimp Penaeus stylirostris in relation with the moult cycle: protection against Vibriosis. Fish Shellfish Immunol. 7: 227-234.
2. Flegel TW (2007) Update on viral accommodation, a model for host-viral interaction in shrimp and other arthropods. Dev Comp Immunol 31: 217-231.
3. Itami T, Yan Y, Takahashi Y (1992) Studies on vaccination against vibriosis in cultured kuruma prawns Penaeus japonicus - I. Effect of vaccine concentration and duration of vaccination efficacy. J Shimonoseki Univ Fish. 40: 83-87.
4. Itami T, Yan Y, Takahashi Y (1992a) Studies on vaccination against vibriosis in cultured kuruma prawns Penaeus japonicus - II. Effect of different vaccine preparations and oral vaccination efficacy. J Shimonoseki Univ Fish. 40: 139-144.
5. Wu JL, Nishioka T, Mori K, Nishizawa T, Muroga K (2002) A time-course study on the resistance of Penaeus japonicus induced by artificial infection with white spot syndrome virus. Fish Shellfish Immunol 13: 391-403.
6. Kurtz J, Franz K (2003) Innate defence: evidence for memory in invertebrate immunity. Nature 425: 37-38.
7. Namikoshi A, Wu JL, Yamashita T, Nishizawa T, Nishioka T, et al. (2004) Vaccination trials with Penaeus japonicus to induce resistance to white spot syndrome virus. Aquaculture. 229: 25-35.
8. Witteveldt J, Cifuentes CC, Vlak JM, van Hulten MC (2004) Protection of Penaeus monodon against white spot syndrome virus by oral vaccination. J Virol 78: 2057-2061.
9. Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61: 243-282.
10. Anderson KM, Srivastava PK (2000) Heat, heat shock, heat shock proteins and death: a central link in innate and adaptive immune responses. Immunol Lett 74: 35-39.

11. Wallin RP, Lundqvist A, Moré SH, von Bonin A, Kiessling R, et al. (2002) Heat-shock proteins as activators of the innate immune system. Trends Immunol 23: 130-135.
12. Picard D (2002) Heat-shock protein 90, a chaperone for folding and regulation. Cell Mol Life Sci 59: 1640-1648.
13. Hartl FU (1996) Molecular chaperones in cellular protein folding. Nature 381: 571-579.
14. Nover L, Scharf KD (1997) Heat stress proteins and transcription factors. Cell Mol Life Sci 53: 80-103.
15. Craig EA, Ingolia TD, Manseau LJ (1983) Expression of Drosophila heat-shock cognate genes during heat shock and development. Dev Biol 99: 418-426.
16. Rybczynski R, Gilbert LI (2000) cDNA cloning and expression of a hormone-regulated heat shock protein (hsc 70) from the prothoracic gland of Manduca sexta. Insect Biochem Mol Biol 30: 579-589.
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.
18. Yoganandhan K, Sathish S, Narayanan RB, SahulHameed AS (2003a) Rapid nonenzymatic method of DNA extraction for PCR detection of white spot syndrome virus in shrimp. Aquat Res. 34:1-5
19. Soderhall K, Smith VJ (1983) Separation of the haemocyte populations of Carcinus maenas and other marine decapods, and prophenoloxidase distribution. Dev Comp Immunol 7: 229-239.
20. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.
21. Ashida M, Soderhall K (1984) The prophenoloxidase activating system in crayfish (Astacus astacus). Dev Comp Immunol. 77: 21-26.
22. Song YL, Hsieh YT (1994) Immunostimulation of tiger shrimp (Penaeus monodon) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species. Dev Comp Immunol 18: 201-209.
23. Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44: 276-287.
24. Sastry KV, Moudgal RP, Mohan J, Tyagi JS, Rao GS (2002) Spectrophotometric determination of serum nitrite and nitrate by copper-cadmium alloy. Anal Biochem 306: 79-82.
25. Sorensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. Ecol Lett. 1025-1037.
26. Kregel KC (2002) Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. Hsp70 and HSF during morphogenesis in the vetigastropod Haliotis asinina. Development Genes and Evolution. 2178: 603-612.
27. Gunter HM, Degnan BM (2007) Developmental expression of Hsp90, Hsp70 and HSF during morphogenesis in the vetigastropod Haliotis asinina. Dev Genes Evol 217: 603-612.
28. Beissinger M, Buchner J (1998) How chaperones fold proteins. Biol Chem 379: 245-259.
29. Li Z, Srivastava P (2004) Heat-shock proteins. Curr Protoc Immunol Appendix 1: Appendix 1T.
30. Arya R, Mallik M, Lakhotia SC (2007) Heat shock genes - integrating cell survival and death. J Biosci 32: 595-610.
31. Loeschcke V, Krebs RA, Dahlgaard J, Michalak P (1997) High-temperature stress and the evolution of thermal resistance in Drosophila. EXS 83: 175-190.
32. Vabulas RM, Wagner H, Schild H (2002) Heat shock proteins as ligands of toll-like receptors. Curr Top Microbiol Immunol 270: 169-184.
33. Morrow G, Tanguay RM (2003) Heat shock proteins and aging in Drosophila melanogaster. Semin Cell Dev Biol 14: 291-299.
34. Huang PY, Kang ST, Chen WY, Hsu TC, Lo CF, et al. (2008) Identification of the small heat shock protein, HSP21, of shrimp Penaeus monodon and the gene expression of HSP21 is inactivated after white spot syndrome virus (WSSV) infection. Fish Shellfish Immunol 25: 250-257.
35. Lo CF, Leu JH, Ho CH, Chen CH, Peng SE, et al. (1996) Detection of baculovirus associated with white spot syndrome virus WSSV in penaeid shrimp using polymerase chain reaction. Dis Aquat Organ. 25: 133-141.
36. Jiang S, Qiu L, Zhou F, Huang J, Guo Y, et al. (2009) Molecular cloning and expression analysis of a heat shock protein (Hsp90) gene from black tiger shrimp (Penaeus monodon). Mol Biol Rep 36: 127-134.
37. Xu W, Mimnaugh EG, Kim JS, Trepel JB, Neckers LM (2002) Hsp90, not Grp94, regulates the intracellular trafficking and stability of nascent ErbB2. Cell Stress Chaperones 7: 91-96.
38. Nathan DF, Vos MH, Lindquist S (1997) In vivo functions of the Saccharomyces cerevisiae Hsp90 chaperone. Proc Natl Acad Sci U S A 94: 12949-12956.
39. Wu LT, Chu KH (2008) Characterization of heat shock protein 90 in the shrimp Metapenaeus ensis: Evidence for its role in the regulation of vitellogenin synthesis. Mol Reprod Dev 75: 952-959.
40. Lee GJ, Roseman AM, Saibil HR, Vierling E (1997) A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. EMBO J 16: 659-671.
41. Jaya N, Garcia V, Vierling E (2009) Substrate binding site flexibility of the small heat shock protein molecular chaperones. Proc Natl Acad Sci U S A 106: 15604-15609.
42. Tassanakajon A, Klinbunga S, Paunglarp N, Rimphanitchayakit V, Udomkit A, et al. (2006) Penaeus monodon gene discovery project: the generation of an EST collection and establishment of a database. Gene 384: 104-112.
43. Lo WY, Liu KF, Liao IC, Song YL (2004) Cloning and molecular characterization of heat shock cognate 70 from tiger shrimp (Penaeus monodon). Cell Stress Chaperones 9: 332-343.
44. Jiravanichpaisal P, Lee BL, Söderhäll K (2006) Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 211: 213-236.
45. Basu S, Binder RJ, Ramalingam T, Srivastava PK (2001) CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity 14: 303-313.
46. Srivastava P (2002) Roles of heat-shock proteins in innate and adaptive immunity. Nat Rev Immunol 2: 185-194.
47. Vega E, Hall M, Degnan B, Wilson K, et al. (2006)Short-term hyperthermic treatment of Penaeus monodon increases expression of heat shock protein 70 (HSP70) and reduces replication of Gill Associated Virus (GAV). Aquaculture. 253: 82-90.
48. Ratclilfe NA, Rowley AF, Fitzgerald SW, Rhodes CP, et al. (1985) Invertebrate immunity: basic concepts and recent advances. INT REV CYTOL. 97: 183-350.
49. Albores VF, Guzman-Murillo A, Ochoa JL, et al.(1993) A lipopolysaccharide-binding agglutinin isolated from brown shrimp Penaeus californiensis Holmes haemolymph. Comp Biochem Physiol. 104A: 407-413.
50. Johansson MW, Keyser P, Sritunyalucksana K, Soderhall K, et al.(2000) Crustacean haemocytes and haematopoiesis. Aquaculture. 191: 45-52.
51. Ourth DD, Renis HE (1993) Antiviral melanization reaction of Heliothis virescens hemolymph against DNA and RNA viruses in vitro. Comp Biochem Physiol B 105: 719-723.
52. Zhang ZF, Shao MY, Kang KH,et al. (2005) Changes of enzyme activity and hematopoiesis in Chinese prawn Fenneropenaeus chinensis (Osbeck) induced by white spot syndrome virus and zymosan A. Aquacult Res. 36: 674-681.
53. Leu JH, Chang CC, Wu JL, Hsu CW, Hirono I, et al. (2007) Comparative analysis of differentially expressed genes in normal and white spot syndrome virus infected Penaeus monodon. BMC Genomics 8: 120.
54. Liu CH, Chen JC (2004) Effect of ammonia on the immune response of white shrimp Litopenaeus vannamei and its susceptibility to Vibrio alginolyticus. Fish Shellfish Immunol. 16:321-334.
55. Escobar JA, Rubio MA, Lissi EA (1996) Sod and catalase inactivation by singlet oxygen and peroxyl radicals. Free Radic Biol Med 20: 285-290.
56. Munoz M, Cedeno R, Rodrýguez J, Van der Knapp WPW, Mialhe E, et al.(2000) Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp, Penaeus vannamei. Aquaculture 191: 89-107.

57. Cheng W, Wang CH (2001) The susceptibility of the giant freshwater prawn Macrobrachium rosenbergii to Lactococcus garvieae and its resistance under copper sulfate. DIS AQUAT ORGAN 47:137-144.