Indian J. Virol. (October–December 2012) 23(3):333–343
DOI 10.1007/s13337-012-0119-7

ORIGINAL ARTICLE

Transcript Analysis of *White spot syndrome virus* Latency and Phagocytosis Activating Protein Genes in Infected Shrimp (*Penaeus monodon*)

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Received: 27 July 2012 / Accepted: 15 October 2012 / Published online: 27 November 2012

Abstract Viral latency has been recently observed to be associated with *White spot syndrome virus* (WSSV) infection in shrimp. In the present study, shrimp samples (*Penaeus monodon*) surviving WSSV infection were examined for presence of WSSV in latent phase. Virus latency was observed in shrimp which were either experimentally challenged with WSSV and survived the infection or those which survived the natural infection. Three viral transcripts (ORFs 427, 151, 366) associated with latency were analyzed by real-time PCR. The shrimp surviving the natural WSSV infection on estimation with RT-PCR were found to have low grade of WSSV infection (less than 56 copies of WSSV). All the shrimp samples were RT-PCR negative for structural protein genes of WSSV, VP24 and VP28, indicating that these samples were harboring latent phase virus. RT-PCR of all the shrimp samples which survived WSSV infection revealed amplification of phagocytosis activating protein (PAP) gene (435 bp) with higher gene expression levels in experimentally challenged shrimp when compared to naturally infected shrimp. The expression of PAP in WSSV infected shrimp samples indicates its possible role in host response for resistance against WSSV infection. PAP was cloned and expressed as recombinant protein for protection studies. Shrimp were injected with three doses (5, 15 and 20 μg g^{-1} body weight) of recombinant PAP. Relative percent survival of 10 % was observed in shrimp immunized with the dose of 15 μg g^{-1} body weight of recombinant PAP. The expression of both WSSV latency associated and PAP genes obtained from shrimp surviving the WSSV infection, indicates the possible role of these genes in host–pathogen interaction.

Keywords *White spot syndrome virus* · *Penaeus monodon* · Latency · Phagocytosis activating protein

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Introduction

Outbreaks of viral disease due to *White spot syndrome virus* (WSSV) in tiger shrimp, *Penaeus monodon*, have been reported since 1994 and continue to be a major problem for the shrimp aquaculture industry worldwide. It has been observed that, despite the prevalence of WSSV in some cases, no mass mortalities were noticed from shrimp ponds with 'light-grade' infections [20] and not all WSSV outbreaks results in crop failure [35]. The tolerance of marine shrimps to other viral diseases has also been reported. For example, multiple viruses such as *Hepatopancreatic parvovirus*, *Monodon baculovirus*, WSSV and *Infectious hypodermal and hematopoietic necrosis virus* (IHHNV) were detected in cultivated shrimp which were free of gross disease signs [10]. Similar observation of existence of WSSV in shrimps in an asymptomatic carrier state has also been reported by Tsai et al. [32]. These observations suggest that either the shrimps were infected with a less virulent, variant strain of the virus or that the affected shrimp had developed some form of resistance to the virus which led to their survival during WSSV outbreaks or against other shrimp viral disease infections.

Viral latency, observed to be associated with DNA viruses, such as *Human cytomegalovirus* [26] and *Epstein–*

Barr virus (EBV) [16] has also been recently reported in case of WSSV infection. While investigating specific-pathogen-free (SPF) shrimp as asymptomatic carriers of WSSV, three WSSV genes (ORFs 366, 151 and 427) were found to be relatively highly expressed in SPF shrimp and to be associated with the latent infection of WSSV [14]. Recently, the transcripts of WSSV ORF 403 could be detected in SPF shrimp, suggesting its role as a latency associated gene. This gene is reported to function as a viral E3 ligase and binds to a shrimp protein phosphatase [13]. Transcriptional analysis of ORF 427 gene suggests that this gene may not have an important role in activating virus replication from latent phase as it is reported to be a late gene during the viral lytic infection. However, ORF 427 possibly contributes to maintaining viral latency by affecting the function of shrimp protein phosphatase [17, 18].

Presently, little information is available about the molecular mechanisms involved in WSSV latent infections and the genes responsible in establishing latent and lytic WSSV infections in shrimp. It is interesting to investigate the role of immune factors and the genes responsible for shrimp resistance against WSSV. In recent years, genes such as phagocytosis activating protein (PAP) [9] and *P. monodon* antiviral gene (*Pm*AV) [19] have been identified which might play an important role in the defense mechanism against WSSV. In the present study, identification of latency associated genes and the host response against WSSV infection has been attempted as an approach to understand the molecular mechanisms involved in host–pathogen interaction in WSSV infected shrimps. We examined the shrimp samples which survived WSSV challenge experiments and natural WSSV infection for presence of latent infection. In order to study the host potential to resist the viral infection, the shrimp samples were also screened for expression of the gene encoding PAP for its possible role in immune response and protection.

Materials and Method

Shrimp

Shrimp (*P. monodon*) which survived WSSV infection during different challenge experiments over a period of time and those surviving the natural infection were collected for the analysis in the present study. The shrimp surviving the experimentally challenged WSSV infection included the samples which were either orally challenged with WSSV infected feed or were intramuscularly challenged with WSSV. The protocol for intramuscular challenge experiments conducted in *P. monodon* shrimp was followed as described previously [11]. Briefly, WSSV viral stock was prepared using infected *P. monodon* tissue which was minced, centrifuged and the resultant supernatant was filtered. After confirming the presence of WSSV by PCR, the viral stock solution was subjected to 10-fold dilutions. Virus dilutions (10^{-1}–10^{-4}) were inoculated (0.1 mL) in *P. monodon* (2–3 g) by intramuscular route. LD_{50} dose ($10^{-3.41}$) was estimated by method of Reed and Muench [22]. This virus dose was subsequently used for different challenge experiments carried out in laboratory. Seven WSSV challenged shrimps collected at different days post-challenge and five shrimps surviving the natural WSSV disease outbreak in shrimp culture ponds were analyzed for WSSV associated latent gene expression as shown in Table 2. The shrimp collected from culture ponds appeared healthy and no apparent symptoms of WSSV infection were observed.

PCR of WSSV Genes from Genomic DNA

Muscle tissues collected from experimentally WSSV challenged and WSSV natural infection surviving shrimp were homogenized in 300 µL of extraction buffer (10 mM Tris (pH 8.0), 25 mM EDTA, 100 mM NaCl, 0.5 % SDS, 100 µg mL^{-1} Proteinase K). The homogenized tissue samples after heating in boiling water for 2 min, were centrifuged at 11,000×*g* for 5 min at room temperature and the supernatant was collected. One microliter of homogenized tissue sample was used as template for nested PCR analysis of WSSV genes. The outer and inner primers for detection of WSSV by nested PCR for 643 and 298 bp were based on the sequence reported by Takahashi et al. [30] (Table 1). The PCR reaction mixture included all 4 dNTPs (200 µM), 30 pmol concentration of each primer, 1 unit of *Taq* polymerase and 1× polymerase buffer containing 1.5 mM $MgCl_2$. The thermal program was carried out with initial denaturation at 93 °C for 2 min followed by 30 cycles of 93 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and 72 °C for 10 min as final extension cycle.

One microliter of homogenized tissue sample was also used as template for real-time PCR analysis (IQ Real™ WSSV quantitative system, Taiwan) following the manufacturer's protocol.

RT-PCR of WSSV Genes and PAP Gene

Total RNA from the muscle tissues of the shrimp samples was extracted using Nucleospin RNA II kit (Macherey–Nagel). After treatment with DNase I, the RNA samples were reverse transcribed using Protoscript first strand cDNA synthesis kit (New England Biolabs) following the manufacturer's protocol. Briefly, the total RNA was reverse transcribed to cDNA at 48 °C for 1 h using random primers and M-MuLV reverse transcriptase. cDNAs were

Table 1 Primer sequences used in amplification of WSSV and PAP genes

Gene	Primer sequence	PCR product size (bp)	Reference/GenBank accession nos.
WSSV1	F: 5′-GACAGAGATATGCACGCCAA-3′	643	[30]
(outer)	R: 5′-ACCAGTGTTTCGTCATGGAG-3′		
WSSV1	F: 5′-GGTAGATTCTGGTATTAGG-3′	298	[30]
(inner)	R: 5′-ACCTGGCGTAGTTCTTGC-3′		
ORF 366	F: 5′-ATGAGGAAAATGACCTCTATGA-3′	252	[14]
(outer)	R: 5′-TCAAGAAAGCGCGTGCTTTAG-3′		
ORF 366	F: 5′-GAGACGTCGCTCATCAAAGATGGGGAAG-3′	160	[14]
(inner)	R: 5′-GAAACCTGGACCATATTGAATACGGCCAG-3′		
ORF 151	F: 5′-ATGGATTTTGAAGGAACTACCA-3′	4300	[14]
(outer)	R: 5′-CTTCTTTGTTTTCTTTG-3′		
ORF 151	F: 5′-GTGGTCACATCTGACAGTGGA-3′	510	[14]
(inner)	R: 5′-GCATAATGCAGTAGCGTCAACGGC-3′		
ORF 427	F: 5′-ATGGCATGGACCGTAATGGC-3′	1870	[14]
(outer)	R: 5′-TTCCTTGATCTAGAGCT-3′		
ORF 427	F: 5′-GAGCTGGCAAAGGAAACC-3′	900	[14]
(inner)	R: 5′-ACAGACAACAGAACCTCCTTC-3′		
VP24	F: 5′-AATAAATCTCTCCCTAACAATGAAAGG-3′	762	[37]
	R: 5′-TTTTCTCTCATGACCTTTGTACAACTT-3′		
VP28	F: 5′-ATGGATCTTTCTTTCACTCT-3′	615	DQ681069
	R: 5′-TTACTCGGTCTCAGTGCCAG-3′		
PAP	F: 5′-CGGCCATGGCTATGAAGATCAATAAG-3′	435	AY680836
	R: 5′-GCCAAGCTTTTAAGATGAGGTGTC-3′		

PAP phagocytosis activating protein

used as templates for PCR amplification of WSSV latency associated genes, structural genes and PAP gene transcripts. Nested PCR was carried out using outer and inner primers amplifying WSSV ORFs 366, 151 and 427 to detect the latency associated WSSV genes as reported by Khadijah et al. [14] (Table 1). Shrimp samples were analysed for WSSV structural genes transcripts VP28 (615 bp) and VP24 (762 bp) using gene specific primers (Table 1). The primers for amplification of full length PAP gene transcripts, were based on the reported gene sequence (GenBank accession no. AY680836). The PCR amplified products of latency associated genes and PAP gene were gel purified (Qiagen) and sequenced using gene specific forward and reverse primers.

PAP mRNA Expression Analysis by real-time PCR

Total RNA extracted using Nucleospin RNA II kit (Macherey–Nagel) from the muscle tissues of the uninfected (control) and WSSV infected shrimp samples was converted to cDNA with cDNA synthesis kit (Protoscript cDNA synthesis kit, New England Biolabs). The cDNA was used to identify the relative expression of PAP transcripts by RT-PCR assay using the Power SYBR Green PCR master mix (Applied Biosystems). The primers were designed with the aid of Primer Express software (Applied Biosystems). The following primers for PAP (F: 5′-AACC GGCAACGATACTTCAG-3′ and R: 5′-TGACGCAGTTC CTTTGATAGG-3′) were used to generate 80 bp PCR product for RT analysis. The shrimp β-actin gene was amplified with the primers (F: 5′-GAACCTCTCGTTGCC GATGGTG-3′ and R: 5′-GAAGCTGTGCTACGTGGCTC TG-3′) to generate 124 bp product which was used as an endogenous control. The relative quantification of the transcripts were assessed by comparative C_T method. The RT-PCR reaction was performed in triplicates in a final volume of 20 μL reaction mixture each. The reaction mixture contained cDNA (10 ng), forward and reverse primer of β-actin or PAP gene (30.0 pmol) and 10 μL of 2× Power SYBR Green PCR master mix with the ROX dye (Applied Biosystems). The reaction conditions for RT-PCR were 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s and 55 °C for 30 s. The PCR amplification was followed by the melt curve conditions of 95 °C for 40 s, 60 °C for 15 s and 95 °C for 15 s. The relative quantification results were expressed as the fold change in levels of the gene expression and statistical analysis of the data for comparison between groups was carried out by one-way ANOVA and the values with $p < 0.05$ were considered significant.

Cloning of PAP Gene and Expression of Recombinant Protein

The protocol for cloning, expression and purification of recombinant protein was followed as described previously [25]. To clone the PAP full length gene fragment, the PAP primers were modified by adding *Nco*I and *Hin*dIII restriction sites to the forward and reverse primers respectively (Table 1). The PCR product containing the restriction sites after restriction enzyme digestion was cloned into the *Nco*I and *Hin*dIII linearized histidine (HIS) tagged pET32a (+) expression vector (Novagen). The plasmid construct was confirmed for the presence of the PAP gene insert by PCR and restriction digestion with *Nco*I and *Hin*dIII restriction enzymes. The plasmid was transformed into *Escherichia coli* strain BL21 (DE3) pLysS for protein expression. The transformed cells were grown in Luria–Bertani medium supplemented with ampicillin until the optical density (OD_{600}) reached 0.6. Protein expression was induced for 4 h by adding 1.0 mM of isopropyl-β-D-thiogalacto-pyranoside (IPTG). Expression of recombinant protein was analysed on SDS-PAGE. The expressed recombinant fusion protein (HIS–PAP) was purified using ProBond nickel chelating resin (ProBond purification system, Invitrogen) following manufacturer's instruction for purification of polyhistidine containing recombinant proteins.

Protection Studies in Shrimp Immunized with Recombinant PAP

Penaeus monodon shrimp (1.52 ± 0.57 g) were divided into eight groups of 30 shrimps each. Three groups were injected with 5 μg g^{-1} (5HIS–PAP), 15 μg g^{-1} (15HIS–PAP) and 20 μg g^{-1} (20HIS–PAP) body weight of recombinant HIS–PAP respectively. Remaining five groups which served as controls, included three groups which were injected with 5 μg g^{-1} (5HIS), 15 μg g^{-1} (15HIS) and 20 μg g^{-1} (20HIS) body weight of HIS protein alone, one group was injected with PBS and the last group served as environmental control. After 3 h, all groups of the shrimps except the environmental control group were challenged by intramuscular injection (100 μL) in the fourth abdominal segment of the shrimp with 10^{-7} dilution of the virus. The 10-fold serial dilutions of the virus was prepared from the virus stock which was estimated to contain 2.62×10^6 μL^{-1} viral copies by real-time PCR analysis. The experiment was carried out in triplicate for the each group of shrimp. The relative percent survival (RPS) was calculated as (1 − infected group mortality/control group mortality) × 100 [1].

After WSSV challenge, survival data was recorded and analyzed for all the experimental shrimp immunized with HIS–PAP recombinant protein in comparison with control groups. Shrimp were observed for mortality at regular intervals and time taken to death post-WSSV challenge was recorded. Mortality in shrimp post-WSSV challenge was recorded for a period of 15 days. Kaplan–Meier (KM) survival curves were generated for experiment and control groups using GraphPad Prism 5.0 windows version and were compared between PBS control group and each dose of HIS and HIS–PAP immunized shrimp groups using log rank test. To find out the effective dose of recombinant HIS–PAP protein conferring better shrimp survival, KM survival curves of three shrimp groups (5, 15 and 20HIS–PAP) were also compared. Survival data was subjected to a cox proportional hazards model to study the influence of covariable (body weight) on the survival using SPSS version 16.

Results

PCR Amplification of WSSV Genes from Genomic DNA

Using the genomic DNA extracted from shrimp samples as template for amplification of WSSV genes, analysis of all the 12 shrimp samples revealed amplification with expected PCR product size (643 bp) using WSSV1 outer primers for two samples (1 and 4). Sample 1 showed a light amplification when compared to sample 4 for WSSV gene using this set of primers (Fig. 1a; Table 2). WSSV1 inner primers revealed amplification with expected PCR product size (298 bp) with varied intensity for 11 samples. PCR product for sample 12 could not be visualized (Fig. 1b; Table 2).

RT-PCR of WSSV Genes and PAP Gene

RT-PCR of the cDNA prepared from the 12 shrimp samples revealed three shrimp samples (1, 4 and 7) to be Ist- (252 bp) and IInd step (160 bp) PCR positive for amplification of ORF 366 whereas, the rest of the shrimp samples were Ist step PCR negative and IInd step PCR positive for ORF 366 (160 bp) using the internal primers of this WSSV gene (Fig. 2a; Table 3). In case of ORFs 151 and 427 the shrimp samples (1–7) were Ist step PCR negative for ORFs 151 (4,300 bp) and 427 (1,870 bp) however, these samples were IInd step PCR positive for ORF 151 (510 bp) (Fig. 2b; Table 3) and ORF 427 (900 bp) using internal primers (Fig. 2c; Table 3). The shrimp samples (8–12) surviving the natural WSSV infection were Ist- and IInd step PCR negative for ORFs 151 and 427. All the shrimp samples were RT-PCR negative for VP24 and VP28 (Table 3). RT-PCR of all the shrimp samples which

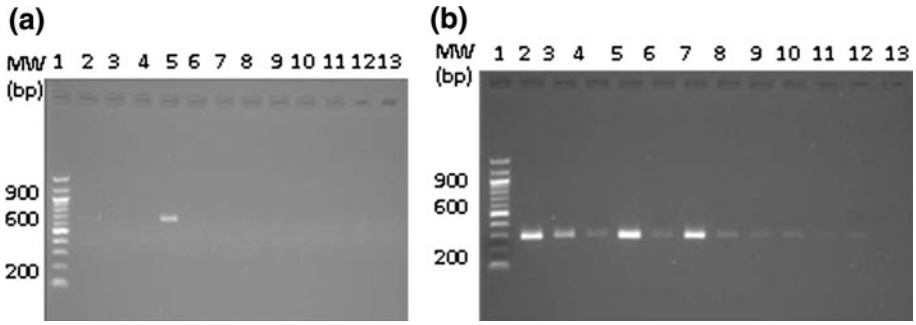

Fig. 1 PCR amplification of WSSV from the genomic DNA extracted from WSSV infected samples. **a** Using WSSV1 outer primers (643 bp). **b** Using WSSV1 inner primers (298 bp). *Lanes 2–13*: samples 1–12

Table 2 Nested PCR analysis in genomic DNA of WSSV infection surviving *P. monodon* shrimp samples

SampleS	Days post-WSSV challenge	Route of WSSV Infection	Nested PCR		WSSV copies
			Ist step (643 bp)	IInd step (298 bp)	
1	28	im	+	+++	5111
2	28	im	–	++	4645
3	28	im	–	+	543
4	28	im	++	+++	32310
5	30	im	–	+	352
6	30	im	–	+++	1327
7	54	Oral	–	+	102
8	–	Natural	–	+	56
9	–	Natural	–	+	47
10	–	Natural	–	+	20
11	–	Natural	–	+	26
12	–	Natural	–	–	20

Sample: Shrimp samples surviving WSSV challenge experiment (1–7) and natural infection (8–12)

Im, intra-muscular injection; nested PCR carried out using WSSV1 outer and inner primers, WSSV copies per μL of the tissue homogenate were determined by real-time PCR using TaqMan assay; +, -light PCR amplification; ++, moderate PCR amplification; +++, strong PCR amplification; –, no PCR amplification

survived WSSV challenge experiments and natural infection revealed amplification of PAP gene (435 bp) (Fig. 2d; Table 3).

Sequence Analysis

The amplicons obtained for latency genes on sequencing showed sequence homology to reported WSSV ORFs 366, 151 and 427 in GenBank, which confirmed that the amplicons corresponded to the three WSSV latency associated genes reported by Khadijah et al. [14]. Sequencing of the 435 bp PAP gene revealed sequence similarity with reported GenBank accession no. AY680836 for *P. monodon* PAP gene.

PAP mRNA Expression Analysis by real-time PCR

Real-time PCR analysis of the shrimp samples exposed to WSSV infection revealed a higher PAP gene expression levels (9.63–47.34-fold) in experimentally challenged shrimp samples when compared to naturally infected samples (2.11–10.30-fold) (Fig. 3).

Cloning and Expression of PAP Gene

The amplification of *P. monodon* PAP gene resulted in expected size of 435 bp PCR product. The positive clones were confirmed for the presence of the insert by PCR and restriction digestion with *Nco*I and *Hin*dIII restriction enzymes (Fig. 4a). On IPTG induction the expression of the fusion recombinant protein was observed, that on purification with ProBond nickel chelating resin revealed purified band with expected size of 33.3 kDa (Fig. 4b).

Protection Studies in Shrimp Immunized with Recombinant PAP

After WSSV challenge, the RPS of the shrimp experimental groups was recorded. The results indicated that 10 % of the shrimp survived in the 15HIS–PAP group. Higher median survival times were observed for the groups of shrimp immunized with recombinant HIS–PAP protein, with highest value recorded for 20HIS–PAP group (Table 4). Log rank tests of KM survival curves (Fig. 5a–d) revealed that HIS–PAP group was significantly different ($p < 0.05$) from the control HIS and PBS groups with their respective doses. No significant differences ($p > 0.05$) were observed between PBS and all three doses of HIS control groups used in this study. With respect to dose of recombinant HIS–PAP required for protection against WSSV, log rank test of KM survival curves revealed no significant difference ($p > 0.05$) between 15 and 20HIS–PAP groups whereas 5HIS–PAP was found significantly different ($p < 0.05$) from both 15 and 20HIS–PAP groups. Cox proportional hazard model revealed that body weight was not influencing the survival of immunized shrimp groups after WSSV challenge (Table 5).

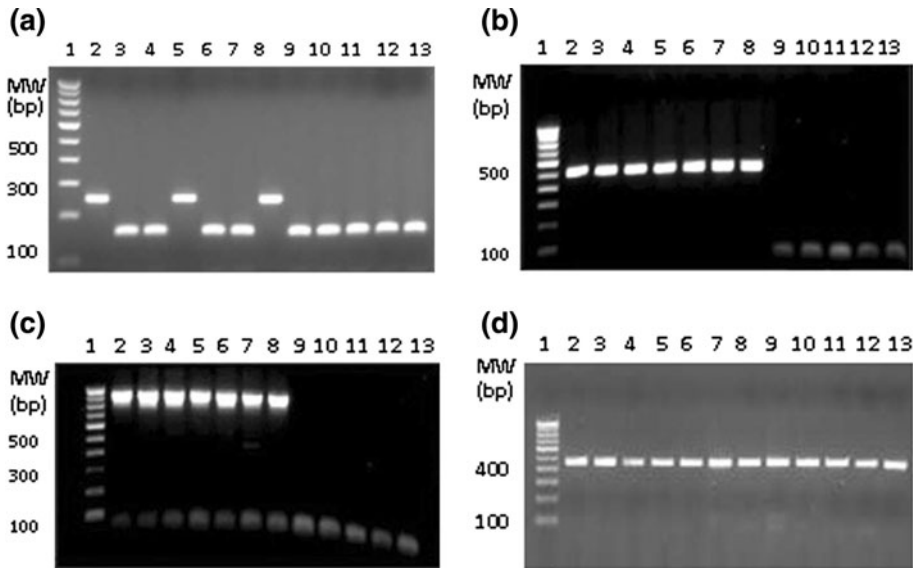

Fig. 2 Expression analysis of WSSV latency associated genes from shrimp samples surviving WSSV infection by RT-PCR **a** ORF 366. *Lane 1* 100 bp marker. *Lanes 2*, *5*, *8* Ist step PCR product (252 bp) for shrimp samples 1, 4, 7 respectively. *Lanes 3*, *4*, *6*, *7*, *9–13* IInd step PCR product (160 bp) for shrimp samples 2, 3, 5, 6, 8–12 respectively. **b** ORF 151. *Lane 1* 100 bp marker. *Lanes 2–8*: shrimp samples (1–7) positive for PCR product (510 bp) respectively. *Lanes 9–13*: shrimp samples (8–12) negative for ORF 151 PCR product respectively. **c** ORF 427. *Lane 1* 100 bp marker. *Lanes 2–8*: shrimp samples (1–7) positive for PCR product of ORF 427 (900 bp) respectively. *Lanes 9–13*: shrimp samples (8–12) negative for ORF 427 PCR product respectively. **d** Expression analysis of PAP gene from shrimp samples surviving WSSV infection by RT-PCR. *Lane 1* 100 bp marker. *Lanes 2–13* PCR product of PAP (435 bp) for shrimp samples 1–12

Table 3 Gene expression analysis in cDNA of *P. monodon* shrimp samples surviving WSSV infection

Gene expression									
Samples	PAP	VP28	VP24	ORF 366 (252 bp)	ORF 366 (160 bp)	ORF 151 (4300 bp)	ORF 151 (510 bp)	ORF 427 (1870 bp)	ORF 427 (900 bp)
1	+	–	–	+	+	–	+	–	+
2	+	–	–	–	+	–	+	–	+
3	+	–	–	–	+	–	+	–	+
4	+	–	–	+	+	–	+	–	+
5	+	–	–	–	+	–	+	–	+
6	+	–	–	–	+	–	+	–	+
7	+	–	–	+	+	–	+	–	+
8	+	–	–	–	+	–	–	–	–
9	+	–	–	–	+	–	–	–	–
10	+	–	–	–	+	–	–	–	–
11	+	–	–	–	+	–	–	–	–
12	+	–	–	–	+	–	–	–	–

Nested PCR was carried out using outer and inner primers amplifying WSSV ORFs 366, 151 and 427 to detect the latency associated WSSV genes. WSSV structural genes transcripts VP28 (615 bp) and VP24 (762 bp) and PAP transcripts were analysed using single step RT-PCR

Sample, shrimp samples surviving WSSV challenge experiment (1–7) and natural infection (8–12); PAP, phagocytosis activating protein; +, positive PCR amplification; –, no PCR amplification

Discussion

Latency is a well studied phenomenon in human herpes viruses such as *Herpes simplex virus type 1* (HSV-1). The latent infections are reported to be established in sensory neurons as a circular episome associated with histones [15]. The active transcription is reported to occur from only one region of the episome encoding the latency associated

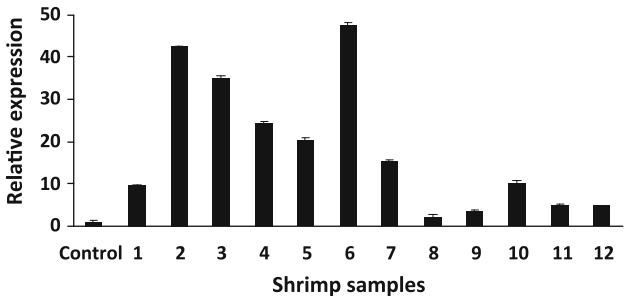

Fig. 3 PAP mRNA expression analysis by real-time PCR in WSSV infected shrimp samples. Shrimp samples (1–7) exposed to WSSV infection by challenge experiment. Shrimp samples (8–12) naturally infected with WSSV

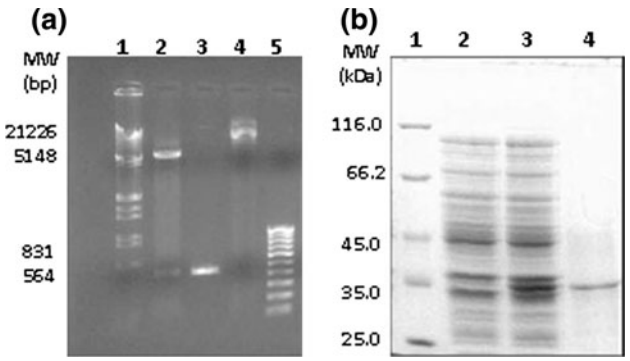

Fig. 4 **a** Restriction enzyme analysis of recombinant plasmids containing PAP gene inserts with *Nco*I and *Hin*dIII restriction enzymes. *Lane 1* Lambda DNA/*Eco*RI/*Hin*dIII double digest marker, *Lane 2* *Nco*I and *Hin*dIII restriction enzyme digested pET32a expression vector. *Lane 3* PAP PCR product (435 bp). *Lane 4* undigested pET32a expression vector. *Lane 5* 100 bp marker. **b** Expression and purification of recombinant PAP. *Lane 1* protein marker. *Lane 2* uninduced cells. *Lane 3* IPTG induced cells showing expression of PAP. *Lane 4* purified PAP

transcript [28, 29]. Cytomegalovirus and EBV are the other two human herpes viruses in which latency has been extensively studied [23, 24]. PCR based reliable, rapid and sensitive systems for detection of latent viral transcripts have been reported for HSV-1 [21], cytomegalovirus [27] and EBV [4]. In case of WSSV infection, very recently, detection of latent genes in WSSV infections has been reported based on RT-PCR and real-time PCR of WSSV ORF 403 in SPF shrimp [13].

Penaeus monodon shrimp harbouring the WSSV virus and surviving the infection is a rare phenomenon and is in contrast to other viral diseases of shrimp in which significant mortalities are not observed in some cases. For example, it has been reported that IHHNV infects *L. vannamei* however, it does not result in significant mortalities [3]. General absence of notable mortalities with the *Yellow head virus* positive shrimp has been observed at the farm level [8]. Cessation of *Taura syndrome virus* (TSV) induced mortalities with resumption of normal behaviour has been observed in chronically infected *P. vannamei* juveniles [12]. The resistance to other shrimp viral infections has even led to development of selected line of *P. stylirostris* postlarvae and juveniles resistant to IHHNV infection [31] and selective breeding of shrimp (*L. vannamei*) for resistance to TSV [2]. In contrast, with highly virulent diseases, such as WSSV, which cause high mortalities of 98 % or more within few days, the frequency of resistance is low and the development of shrimp resistant lines for disease resistance against WSSV is not yet successful [7].

Nested PCR amplification from genomic DNA of infected shrimps for WSSV genes (643 and 298 bp), showed that the shrimp samples analysed in this study were exposed to WSSV infection. The intensity of the amplified

Table 4 Median survival times along with standard errors (SE) of estimates and 95 % confidence intervals (95 % CI); and percentiles (25 and 75) for all experimental groups

Group	Percentile 25		Median survival time			Percentile 75	
	Estimate	SE	Estimate	SE	95 % CI	Estimate	SE
PBS	4.35	0.11	2.90	0.50	1.91–3.88	2.00	0.05
5HIS	3.98	0.31	2.65	0.34	1.99–3.30	2.10	0.56
15HIS	3.77	0.26	2.10	0.24	1.64–2.57	2.04	0.07
20HIS	3.77	0.12	2.77	0.28	2.22–3.32	2.29	0.16
5HIS–PAP	6.21	0.14	4.65	0.51	3.65–5.64	4.17	0.98
15HIS–PAP	8.08	1.21	4.65	0.82	3.04–6.25	3.77	0.85
20HIS–PAP	7.79	0.35	6.73	0.29	6.17–7.29	4.65	0.87
Overall	5.65	0.23	3.85	0.24	3.38–4.32	2.10	0.10

Three shrimp groups were injected with 5 μg g^{-1} (5HIS–PAP), 15 μg g^{-1} (15HIS–PAP) and 20 μg g^{-1} (20HIS–PAP) body weight of recombinant HIS–PAP respectively. Remaining four groups which served as controls, included three groups which were injected with 5 μg g^{-1} (5HIS), 15 μg g^{-1} (15HIS) and 20 μg g^{-1} (20HIS) body weight of HIS protein alone and one group was injected with PBS

Median survival time the time with 0.5 probability of survival, *percentile 75* the time at which 75 % of shrimp are still alive, *percentile 25* the time at which 25 % of shrimp are still alive, *95 % CI* the interval in which the true value of median survival time lies, with 95 % confidence

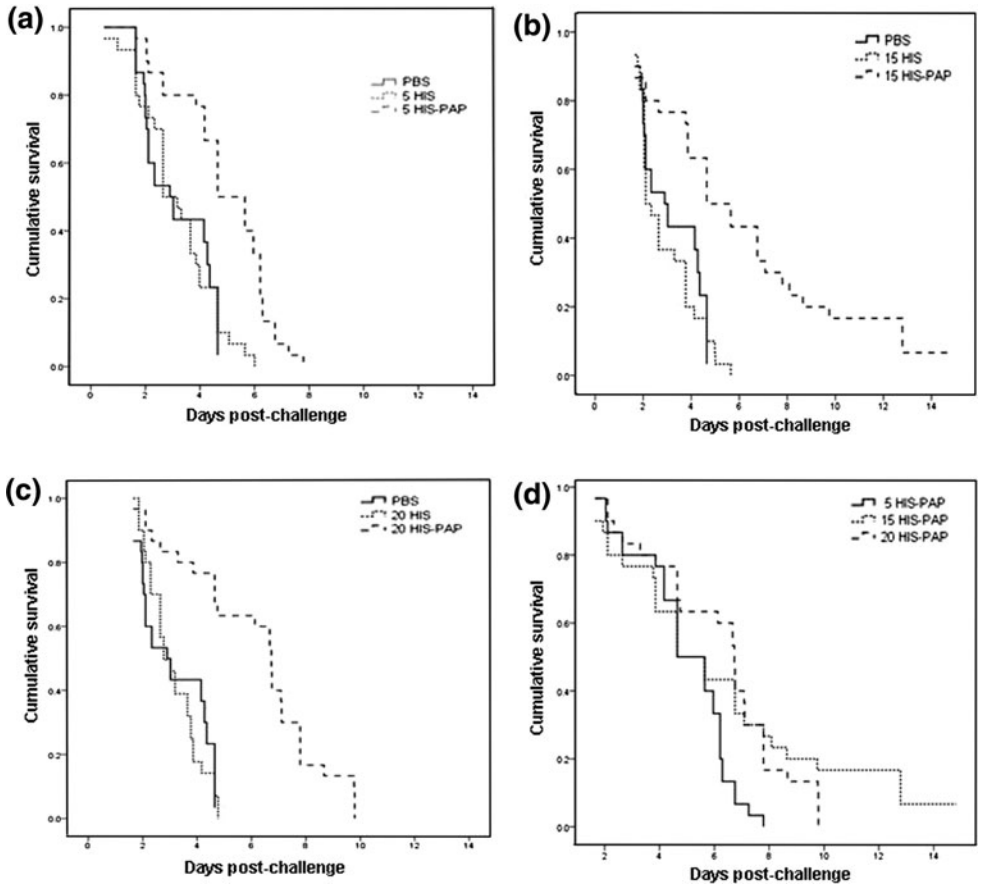

Fig. 5 Log rank tests of KM survival curves (**a**). KM survival analysis between 5HIS–PAP and control groups (5HIS and PBS) (**b**). KM survival analysis between 15HIS–PAP and control groups (15HIS and PBS) (**c**). KM survival analysis between 20HIS–PAP and control groups (20HIS and PBS) (**d**). KM survival analysis between 5, 15 and 20HIS–PAP groups

nested PCR products could be attributed to the WSSV copy numbers present in infected tissue (Fig. 1a, b; Table 2). The shrimp samples which were experimentally challenged with WSSV and survived the infection relatively harbored more viral copy numbers than the other shrimp which survived the natural infection. The naturally infected shrimp samples harbored less than 56 copies of the virus (Table 2). Although in all the WSSV infected shrimp samples, the transcripts for WSSV structural genes (VP28 and VP24) when analysed by RT-PCR were below detection level, however, the transcripts of WSSV latent genes (ORFs 366, 151 and 427) could be detected. Similar observations has been reported, where the three WSSV latency associated genes were found to be relatively highly expressed in SPF shrimps [14]. Asymptomatic carriers have been reported for other shrimp viral diseases also. For example, *L. vannamei* were shown to be asymptomatic carriers of IHHNV [3]. Studies confirming the establishment of virus latency in viral diseases other than WSSV would help in better understanding of the mechanism of viral latency in shrimp in general.

Khadijah et al. [14] used nested PCR to detect the three WSSV latent genes (ORFs 151, 427 and 366) from cDNA reverse transcribed from SPF shrimps. They reported the use of primers amplifying full length WSSV ORFs 151 (4.3 kb), 427 (1.87 kb), and 366 (252 bp) in the first step PCR in the presence of a higher magnesium chloride concentration followed by nested PCR using inner primers for these genes ORFs 151 (510 bp), 427 (900 bp), 366 (210 bp). Interestingly, we obtained an amplified product of 160 bp using internal primers described for ORF 366, whereas, Khadijah et al. [14]. reported an amplification of 210 bp size PCR product using these primers. On alignment of these internal primer sequences of ORF 366 with the three completely sequenced WSSV genome information available at GenBank from the virus isolates of Taiwan (NCBI accession number AF440570), China (NCBI accession number AF332093; [36] and Thailand (NCBI accession number AF369029; [33], we observed that use of these internal primers for ORF 366 should result in amplification of PCR product size of 160 bp. Hence, the amplification of 160 bp obtained in the present study using internal primers for ORF 366 appears correct.

Table 5 Results of Cox's regression for different doses of HIS–PAP tested using group and weight as explanatory variables

Variable	Coefficient (b)	SE	*p* value	e^b	95 % CI for e^b
PBS, 5HIS and 5HIS–PAP (5HIS–PAP as reference group)					
Group			0.00		
PBS	1.62	0.35	0.00	5.06	2.57–9.94
5HIS	1.47	0.33	0.00	4.36	2.28–8.30
Weight	0.19	0.21	0.37	1.21	0.80–1.83
PBS, 15HIS and 15HIS–PAP (15HIS–PAP as reference group)					
Group			0.00		
PBS	1.28	0.33	0.00	3.60	1.90–6.82
15HIS	1.34	0.32	0.00	3.82	2.03–7.22
Weight	−0.21	0.19	0.29	0.81	0.56–1.19
PBS, 20HIS and 20HIS–PAP (20HIS–PAP as reference group)					
Group			0.00		
PBS	1.80	0.40	0.00	6.03	2.78–13.08
20HIS	1.89	0.40	0.00	6.64	3.05–14.44
Weight	−0.39	0.24	0.10	0.68	0.43–1.07
5, 15 and 20HIS–PAP (20HIS–PAP as reference group)					
Group			0.04		
5HIS–PAP	0.55	0.29	0.05	1.74	0.99–3.04
15HIS–PAP	−0.15	0.29	0.59	0.86	0.49–1.50
Weight	−0.16	0.18	0.37	0.85	0.60–1.22

Three shrimp groups were injected with 5 μg g^{-1} (5HIS–PAP), 15 μg g^{-1} (15HIS–PAP) and 20 μg g^{-1} (20HIS–PAP) body weight of recombinant HIS–PAP respectively. Remaining four groups which served as controls, included three groups which were injected with 5 μg g^{-1} (5HIS), 15 μg g^{-1} (15HIS) and 20 μg g^{-1} (20HIS) body weight of HIS protein alone and one group was injected with PBS

SE standard error of coefficient 'b', *p value* indicates statistical significance, e^b exponential or antilog of coefficient 'b', *95 % CI for* e^b the interval in which the true value of e^b lies, with 95 % confidence

The transcripts for ORF 366 using either the external or internal primers could be detected by nested PCR in cDNA of the shrimp samples surviving from the natural WSSV outbreak. In these shrimp samples amplification for the other two latency associated genes (ORFs 151 and 427) could not be obtained from the cDNA. To achieve amplification for ORFs 151 and 427, varied PCR conditions were attempted. In addition to random primers, oligo-dT primers were tried in these samples to generate cDNA templates. The PCR amplification reactions included 1.5–2.0 units of *Taq* DNA polymerase in order to obtain an adequate enzymatic activity. The concentrations of $MgCl_2$ in the range of 1.5–6.0 mM with varied annealing temperature were used to optimize the PCR reaction conditions. Increase in PCR elongation step up to 3 min with overall increase in the number of PCR cycles up to 40 cycles were also attempted as Khadijah et al. [14]. reported use of more number of PCR cycles to detect the expression of latent transcripts. Priming of RT reactions with oligo-dT and random hexamers are commonly used techniques, However, it appears that detection for ORFs 151 and 427 WSSV latency transcripts in samples (8–12) having less copy number of virus estimated by real-time PCR (Table 2) may require modifications in the RT-PCR protocol. For example, as in case of EBV latency detection, the EBV-specific RT-PCR, oligo-dT and random hexamer priming of cDNA synthesis was reported to be less sensitive than multi-priming method [5].

Penaeus monodon PAP a highly homologous gene to the ribosomal protein L26 (RPL26) from *Marsupenaeus japonicus* (98.6 %) [34] and similar to RPL26 from *Mus musculus* (63.1 %) has been isolated from the haemolymph of a WSSV infected shrimp [9]. This gene although homologous to RPL26, was named after its phagocytic activation activity in shrimp [9]. Expression of PAP in *P. monodon* has been reported to be induced with immunostimulants, suggesting that PAP could be a part of a general immune response [9]. We observed the expression of PAP gene in shrimps surviving WSSV challenge experiments and natural infection. The expression of PAP in *P. monodon* haemolymph has been reported to get induced by an intramuscular injection of formalin-inactivated WSSV with increased expression as early as 24 h post-injection which continued to increase during week 1 until week 2 time interval [6]. The RT-PCR in the present study showed expression of PAP in the muscle tissues of all the WSSV infected shrimps which had survived the viral infection. However, the experimentally challenged shrimp samples revealed a higher PAP gene expression levels when compared to naturally infected samples which may be due to virus quantity present in infected shrimp samples. Real-time PCR analysis revealed high WSSV viral copy numbers in experimentally challenged shrimps when compared to shrimps which survived natural infection in the farm with very low viral copies (Table 2). It has been hypothesized that WSSV disease outbreaks may not occur if shrimp defense mechanisms can manage to contain low intensity viral infections under low-stress culture conditions [32].

Highest median survival time was observed for recombinant protein (HIS–PAP) administered shrimp groups compared to control groups (HIS and PBS). The significant difference observed between HIS–PAP groups from HIS and PBS control groups as revealed by KM survival curves with the observed extended survival time in HIS–PAP groups indicates that the administered recombinant HIS–PAP may have a role in delaying mortalities occurring due to WSSV infection. Cox proportional hazard model revealed that shrimps immunized with 5HIS–PAP dose have higher risk of dying (1.74 times) compared to shrimps immunized with 20HIS–PAP.

The use of recombinant PAP protein has been reported to result in protection of the shrimps from WSSV infection.

The shrimps injected with GST-PAP (4, 16 and 32 μg g^{-1} body weight) when challenged with a 9×10^{-6} WSSV stock solution, revealed RPS of 13 %, 64 % and 33 %, respectively. The dose of PAP that gave the best protection was 16 μg g^{-1} body weight in *P. monodon* shrimps reported to be in body weight range of 10–15 g [6]. In the present study the best protection of 10 % was offered by the PAP dose at 15 μg g^{-1} body weight in *P. monodon* shrimps which were having average body weight of 1.6 g.

In conclusion, our study demonstrates that it is feasible to detect WSSV latency associated gene (ORF 366) in either Ist- or IInd step nested RT-PCR and ORFs 151, 427 in the IInd step RT-PCR in the shrimps. ORF 366 (160 bp) could be detected in shrimp samples which harbored as low as 56–20 copies of WSSV. The absence of transcripts for the WSSV structural genes VP24 and VP28 and detection of latency associated genes implies that WSSV latency associated gene transcription takes place in asymptomatic shrimps which survives the WSSV infection. The samples 1, 4 and 7 (Table 3) on comparison were found to be single step negative for structural genes (VP28 and VP24) and Ist step positive for latent gene ORF 366. However, the conventional one-step RT-PCR used for detection of structural genes may not be sensitive enough to detect very low copy numbers of these viral genes. It may be necessary to further confirm by nested PCR or to use amplified RNA as reported by Khadijah et al. [14].

The expression of PAP gene obtained from shrimps surviving the WSSV infection, indicates that PAP may be one of the factors involved in host immune response. However, the molecular mechanisms regulating WSSV latency and reactivation, during natural WSSV infection, remains poorly understood and require extensive investigations. Elucidation of the molecular mechanisms for latency in WSSV infected cells should give new insight for the host cell–virus interaction and facilitate the development of specific anti-viral therapy.

Acknowledgments The authors are thankful to Mr. S. Santhana Krishnan for providing the shrimp samples from the farms which had survived the WSSV natural infection for this study.

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