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A quantitative study on the relative virus load of white spot syndrome virus in infected tissues of tiger shrimp *Penaeus monodon*

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A combination of molecular diagnostic techniques such as nested PCR, real time PCR, Digoxigenin (DIG) probe based *in situ* hybridization and dot blot were used to determine the virus load, tissue tropism and the viral spread in WSSV infected *Penaeus monodon*. Gills, gut, eye stalk, haemolymph, pleopod, muscle and hepatopancreas of WSSV infected shrimp collected at different time post infection period on analysis indicated gill tissues to be the primary target site for WSSV replication at early infection stage. Highest WSSV copies were observed in gill tissue of the infected shrimp at all time points post WSSV infection. DIG labelled 615 bp WSSV VP28 probe was able to detect WSSV in all the infected shrimp tissues. The highest intensity of the colour development by DNA dot blot was observed in infected gills, gut and muscle tissues of the shrimp. Histopathological observations by *in situ* hybridization of gill sections from WSSV infected *P. monodon* revealed progressive increase of positive hybridization signals with the increase in disease duration from 6 h to moribund stage of infection. These results indicate that the virus is able to replicate over time and WSSV pathogenesis proceed with increase in viral copy numbers with disease duration from 6 h to moribund stage in primary target tissue of WSSV infection such as gills.

[Keywords: WSSV, *Penaeus monodon*, RT-PCT, dot blot, *in situ* hybridization]

Introduction

Viral disease due to white spot syndrome virus (WSSV) in shrimp results in severe economic loss to the shrimp aquaculture industry worldwide. The disease leads to large scale mortalities which occur rapidly within 3 to 10 days in shrimp culture[1]. Good management practice as a control measure is advocated with emphasis for early detection of the virus by sensitive and accurate molecular diagnosis, which helps in maintaining healthy brood stock and in production of good quality shrimp larvae. It is important to have better understanding of the viral pathogenesis which would help in developing effective diagnostic tests for various infective stages of the virus. In addition, understanding the aspects of viral pathogenesis would lead to identify the main targets of WSSV, primary site of viral infection and disease progression. In *Penaeus monodon*, stomach, gills, hematopoietic tissue, lymphoid organ, antennal gland and cuticular epidermis were observed to be severely infected by the virus[2]. Whereas, gills, the foregut, integument and antennal gland were observed to be the main WSSV target organs in *Litopenaeus vannamei*[3]. All these organs are of ectodermal or mesodermal origin and WSSV is known to infect cells of these origin[4]. In spite of extensive studies carried out on WSSV pathogenesis, the insufficient experimental evidence, varying experimental conditions and inconsistent results have led to controversies regarding the WSSV entry sites, virus replication and the spread of virus infection to target organs. Disease progress of WSSV in shrimps still remains unclear mainly due lack of stable shrimp cell lines.

Some of the various diagnostic techniques that have been developed to detect WSSV, includes DNA probe based *in-situ* hybridization[2,5], polymerase chain reaction[6-7], histopathology[8] and Real time PCR[9]. Present study is an attempt to study WSSV pathogenesis using a combination of molecular and histological techniques to co-relate the virus load and tissue tropism of WSSV infected shrimp from early stage (6 h) to late (moribund) stage of viral infection.

Materials and Methods

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 stock was estimated to contain 2.62×10^6 εL^{-1} viral copies by real time analysis. A total of 90 *P. monodon* shrimp (average body weight 30 g) were used in this experiment. Shrimps were further divided in 5 groups of 18 number each in 5 tanks of 100 L capacity. The four groups of shrimps were challenged with 0.1 mL of virus (10^{-7} dilution) by

intramuscular route. Gills, gut, eye stalk, haemolymph, pleopod, muscle and hepatopancreas were collected from 6 shrimps from WSSV infected group at each time point of 6 h, 24 h, 48 h and post 48 h upto 72 h (moribund stage) post infection period. The fifth group of shrimps was maintained as uninfected control. Tissue samples and hemolymph was used for further analysis by PCR, real time PCR and histopathology studies.

Shrimp tissues collected at different time intervals post WSSV infection were homogenized in 300 εL of extraction buffer (10 mM Tris (pH 8.0), 25 mM EDTA, 100 mM NaCl, 0.5% SDS, 100 µg mL^{-1} Proteinase K). Homogenized tissue samples after heating in boiling water for 2 min, was centrifuged at 11000 X g for 5 min at room temperature and the supernatant was collected. Supernatant was used for phenol chloroform extraction, the DNA was ethanol precipitated and dissolved in TE buffer. The concentration of DNA extracted from WSSV infected shrimp tissue samples was estimated using nandrop (Nanodrop 2000C, Thermo scientific, USA) for use as template in nested WSSV PCR, real time PCR and dot blot.

Nested WSSV PCR was carried out in infected tissue samples of six shrimp collected at different time intervals. The outer (F: GACAGAGATATGCACGCCAA R: ACCAGTGTTTCGTCATGGAG) and inner primers (F: GGTAGATTCTGGTATTAGG R: ACCTGGCGTAGTTCTTGC) for detection of WSSV by nested PCR for 643 bp and 298 bp respectively were based on the reported sequence of WSSV[7]. The PCR reaction mixture included all 4 dNTPs (200 µM), 30 pico moles concentration of each primer, 1 unit of Taq polymerase and 1X polymerase buffer containing 1.5 mM $MgCl_2$ and 1 εL of DNA (100 ng) extracted from WSSV infected shrimp tissue samples collected at different time intervals as template. 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 1min and 72 °C for 10 min as final extension cycle.

Real time PCR analysis was carried out using IQ REAL ™ WSSV TaqMan assay following manufacturerôs instructions. Briefly, 10.5 εL of real time premix (reaction buffer, dNTPs, specific primers, TaqMan probe) and 1 εL of IQzyme DNA polymerase (2U $εL^{-1}$) was mixed with 1 εL of template (genomic DNA (100 ng) extracted from infected shrimp tissues at different time intervals). The PCR cycling conditions include at 42 °C for 30 sec, followed by 40 cycles of 93 °C for 15 sec and 60 °C for 1 min. The relative expression values were obtained by comparison with the standard provided in the kit. The WSSV copy numbers was quantified per εL of DNA extracted from infected shrimp tissues

PCR was performed by using WSSV VP28 gene specific primers (F: ATGGATCTTTCTTTCACTCT R: TTACTCGGTCTCAGTGCCAG) based on GenBank accession no (DQ 681069) in a 50 εL PCR reaction mixture. The PCR reaction mixture included all 4 dNTPs (200 µM), 30 pico moles concentration of each primer, 1 unit of Taq polymerase and 1X polymerase buffer containing 1.5 mM $MgCl_2$. The PCR reaction mixture was mixed with 1 εL of template (genomic DNA extracted from infected gill shrimp tissue) 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 1min and 72 °C for 10 min as final extension cycle. The PCR product of VP28 (615 bp) was gel purified using QIAquick gel extraction kit (Qiagen, Germany) before being used for DIG labelling.

For preparation of WSSV probe, DIG DNA labelling and detection kit (Roche Applied Science, Germany) was used following the manufacturerôs instructions. Briefly, 15 εL of VP28 purified PCR product (1 µg) was denatured for 10 min. The denatured DNA product was chilled on ice for 2 min. To the denatured DNA, 2 εL of hexanucleotide mix (10X), 2 εL of dNTP labelling mix and 2 εL of Klenow enzyme were gently mixed and briefly centrifuged. The reaction mixture was incubated at 37 ºC for overnight and the reaction was stopped by heating at 65 °C for 10 min. The labelling efficiency of the DIG labelled WSSV VP28 probe was analyzed by comparing the intensity of the colour development of the experimental probe with the control labeled DNA provided in the kit.

The DNA extracted from WSSV infected gills, gut, eye stalk, haemolymph, pleopod, muscle and hepatopancreas collected at moribund stage and from control uninfected group of shrimp were used for DNA dot blot. The experiment was carried out using DIG DNA labelling and detection kit (Roche Applied Science, Germany) following the manufacturerôs instructions. Briefly, 1 εL of undiluted and 10 fold dilutions of the genomic DNA (100 ng) extracted from WSSV infected tissues and control shrimp were spotted onto nylon membrane. The spotted DNA were fixed on the nylon membrane by UV cross linking. The membrane was incubated with 10 mL of 1X

prehybridization buffer (Merck, India) for 30 min followed by incubation overnight in 10 mL of 1X hybridization buffer (Merck, India) containing 1.5 εL VP28 DIG labelled probe. The membrane was washed in washing buffer (2X SSC, 0.1% SDS) twice for 5 min each followed by further washing (0.5X SSC, 0.1% SDS) twice for 15 min each. The membrane was rinsed in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5), followed by incubation with blocking solution (1X) for 30 min and in antibody solution (1:5000 anti Digoxigenin- Alkaline phosphatase (AP) conjugate in blocking solution) for 30 min. The membrane was washed twice with washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% Tween 20) for 15 min each. The membrane was developed using colour substrate solution (BCIP/NBT) in detection buffer (0.1 M Tris, 0.1 M NaCl, pH 9.5).

Gills and hepatopancreas isolated from WSSV infected shrimp at 6 h, 24 h, 48 h and moribund stage along with tissues of uninfected (control) sample were immediately fixed in freshly prepared Davidsonôs fixative for 48 h at room temperature. The tissues were then transferred into 70% alcohol and dehydrated using 90% alcohol for 45 min and in 100% alcohol twice for 30 min each at room temperature. The tissues were treated in alcohol/acetone mixture (50:50) for 30 min and in 100% acetone for 45 min. This was followed by incubation in acetone/xylene mixture (50:50) for 30 min and finally in xylene at room temperature. The dehydrated tissues were immersed into pre-warmed molten paraffin wax and incubated at 60 °C for 30 min. Tissue wax blocks were prepared in embedding system (Spencers, India) for transverse sectioning of the tissues of 5 µm thickness. The sections were placed onto plain glass slides and were stained with haematoxylin and eosin (H&E stain) for histology studies. For *in situ* hybridization, the sections were placed onto positively charged poly-L- lysine (Polysciences Inc., USA) and the slides were allowed to dry overnight.

The tissue sections were heated on hot plate at 65 °C for 30 min. The sections were deparaffinized twice for 30 min each in xylene and rehydrated by incubating in series of graded alcohol solutions of 100%, 80% and 50% for 30 min each and finally rinsed with distilled water. Tissue sections were treated with 100 µg mL^{-1} proteinase K in TNE (50 mM Tris, 50 mM NaCl, 5 mM EDTA) buffer at 37 °C for 30 minutes. Slides were post fixed by chilling in pre-cooled 0.4 % formaldehyde for 5 min at 4 °C and washed in 2X sodium chloride sodium citrate buffer (1X SSC = 150 mM NaCl, 15 mM Tri-sodium citrate, pH 7.0) at room temperature. The slides were incubated with 1X prehybridization buffer (Merck, India) for 30 min at 42 °C. The DIG labelled VP28 WSSV probe was diluted in 1X hybridization buffer (Merck, India) to a final concentration of 100 ng mL^{-1}. For each slide, 500 µL of hybridization solution containing the probe was added. The slides were kept on a hot plate at 95 °C for 6 min and immediately put on ice for 5 min. The probe was allowed to hybridize in a hybridization oven for 16-20 h at 42 °C.

After hybridization, the sections were washed by incubating in washing buffer (2X SSC, 0.1% SDS) for 15 min, (1X SSC, 0.1% SDS) for 5 min (twice) and in 0.5X SSC, 0.1% SDS for 5 min (twice) at 37 °C. This was followed by a wash with maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 5 min at room temperature. The slides were then incubated with blocking solution for 30 min at 37 °C. Anti-DIG - AP -conjugated antibody solution (1:5000 anti Digoxigenin -AP conjugate in blocking solution) was added to each slide and incubated at 37 °C for 30 min. Sections were then washed twice for ten minutes in washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% Tween 20) and once with detection buffer (0.1 M Tris, 0.1 M NaCl, pH 9.5) at room temperature. Slides were developed using 500 µL of colour substrate NBT/BCIP solution in detection buffer at room temperature. The reaction was stopped by washing the slides with TE buffer. Finally the slides were washed by dipping in distilled water and counter stained with 0.5% aqueous Bismarck Brown Y (Sigma) for 2 min and rinsed with distilled water. The sections were then mounted and viewed through a microscope and photographs taken.

Results and Discussion

In this study, all the infected shrimp tissues were found to be either 1st step (643 bp) or 2nd step (298 bp) PCR positive for WSSV at different time intervals post WSSV infection. Among the shrimp tissue samples collected at 6 h, one shrimp gill sample showed positive reaction for WSSV in the first step (643 bp) indicating gill tissues to be the primary target

Table 1. WSSV nested PCR of WSSV infected shrimp (*P. monodon*) tissue samples at different time intervals post WSSV infection

	6 h		24 h		48 h		Moribund	
	1st step	2nd step	1st step	2nd step	1st step	2nd step	1st step	2nd step
Gills	1/6	6/6	2/6	6/6	6/6	6/6	6/6	6/6
Gut	0/6	6/6	0/6	6/6	6/6	6/6	6/6	6/6
Hemolymph	0/6	6/6	0/6	6/6	6/6	6/6	6/6	6/6
Pleopod	0/6	6/6	0/6	6/6	6/6	6/6	6/6	6/6
Eyestalk	0/6	6/6	0/6	6/6	6/6	6/6	6/6	6/6
Muscle	0/6	6/6	1/6	6/6	6/6	6/6	6/6	6/6
Hepatopancreas	0/6	6/6	0/6	6/6	1/6	6/6	3/6	6/6

site for WSSV replication at early infection stage. At 24 h, 2 shrimp gill samples and 1 muscle sample were found first step positive. By 48 h, all the tissues and one of the shrimp hepatopancreas sample was found positive for the first step PCR. In the moribund stage, all tissues showed positive in the first step, except for three shrimp hepatopancreas samples that were positive only in the second step of the PCR (Table 1).

We, observed that hepatopancreas of WSSV infected shrimp were not 1st step PCR positive till 24 h post WSSV infection and hepatopancreas from 3 shrimp samples remained 1st step PCR negative even at the moribund stage of shrimp. The high and low levels of WSSV infection in gills and hepatopancreas respectively as revealed by nested PCR is in agreement with the other studies. Earlier reports using PCR to determine the pattern of WSSV virus multiplication in tissues of shrimp have indicated that WSSV was particularly prevalent in the gills with relatively less prevalence in the hepatopancreas. Lightly infected *P. monodon* revealed 78% and 21% prevalence of WSSV in gill and hepatopancreas respectively by 2nd step PCR. Whereas, WSSV tissue tropism in heavily infected *P. monodon* revealed 100% and 50% prevalence of WSSV in gill and hepatopancreas respectively by 1st step PCR[10] . In the present study, hemolymph of all 6 shrimps tested positive at 6 h and 48 h by 1st and 2nd step PCR respectively. This time range by which presence of WSSV became detectable by PCR is in agreement with the results obtained by others who reported WSSV PCR positive reaction starting at 12 hpi in the cell-free hemolymph in shrimps infected with high dose of WSSV using 1st step PCR[3].

Similar results were obtained in real time quantification of WSSV copies in infected *P. monodon* tissues (Fig. 1, Table 2). Highest WSSV copies were observed in gill tissue of the infected shrimp at all time points post WSSV infection. At moribund stage, gill tissues contained highest viral copies (3.0×10^7) and hepatopancreas contained the lowest viral copies (2.4×10^6). Our observation of gut tissues at moribund stage to be the highly infected tissue (2.7×10^7) after gills is in agreement with other reports which showed foregut epithelia to be the primary target of WSSV infection[11] . Overall grading in decreasing order of the viral load from gills, gut, muscle hemolymph, eyestalk, pleopod and hepatopancreas observed in this study is similar to the results obtained by others who have reported distribution of WSSV in most of the ectodermal and mesodermal organs of experimentally infected shrimp at early stage (12 hpi) of WSSV infection[12].

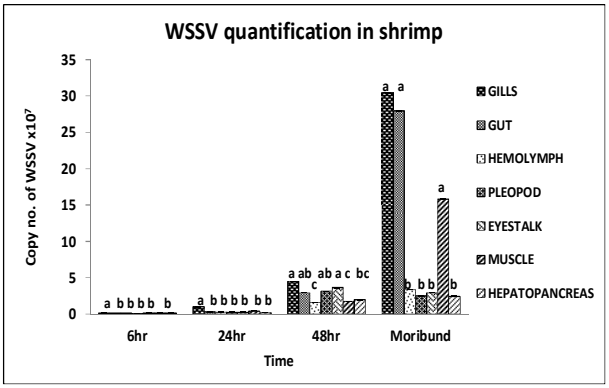

Figure 1. Real time PCR analysis of WSSV viral load in infected shrimp (*P. monodon*) tissues

Table 2. Quantification of WSSV in infected shrimp (*P. monodon*) tissue samples at different time intervals post WSSV infection by Real time PCR analysis

	6 h	24 h	48 h	moribund
Gills	$1.4x10^5 \pm 0.27$	$9.8\ x10^5 \pm 0.04$	$4.4\ x10^6 \pm 0.25$	$3.0\ x10^7 \pm 0.08$
Gut	$1.0\ x10^5 \pm 0.60$	$2.9\ x10^5 \pm 0.71$	$2.8\ x10^6 \pm 0.08$	$2.7\ x10^7 \pm 0.52$
Hemolymph	$1.0\ x10^5 \pm 0.37$	$2.7\ x10^5 \pm 1.1$	$1.5\ x10^6 \pm 0.06$	$3.3\ x10^6 \pm 0.07$
Pleopod	$8.5\ x10^4 \pm 1.48$	$2.3\ x10^5 \pm 0.04$	$3.1\ x10^6 \pm 0.61$	$2.5\ x10^6 \pm 0.41$
Eyestalk	$1.4\ x10^5 \pm 0.46$	$1.9\ x10^5 \pm 0.75$	$3.6\ x10^6 \pm 0.2$	$2.9\ x10^6 \pm 0.88$
Muscle	$1.3\ x10^5 \pm 0.91$	$4.2\ x10^5 \pm 0.23$	$1.7\ x10^6 \pm 0.36$	$1.5\ x10^7 \pm 0.07$
Hepatopancreas	$1.2\ x10^5 \pm 0.39$	$1.8\ x10^5 \pm 0.41$	$1.9\ x10^6 \pm 0.37$	$2.4\ x10^6 \pm 0.97$

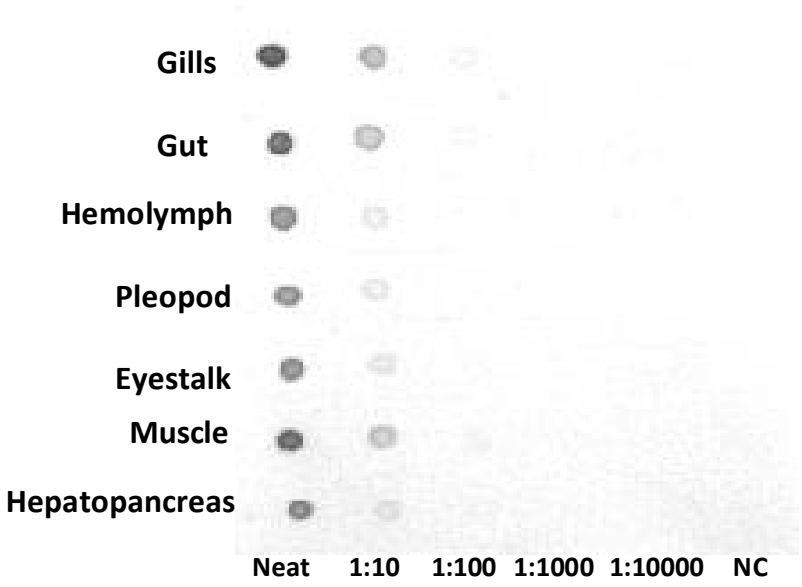

Figure 2. Detection of WSSV in infected shrimp tissues using DIG labelled WSSV probe

The DIG labelled WSSV probe was able to detect WSSV in all the infected shrimp tissues. The highest intensity of the colour development by DNA dot blot was observed in infected gills, gut and muscle tissues of the shrimp. The positive colour development was observed in these tissues at 1:100 dilution of the genomic DNA (Fig. 2). These results are consistent with our previous study in which we reported successful detection of WSSV in different infected tissues of *P. monodon* using DIG labelled WSSV probe[13].

Histopathological changes, in the form of typical intranuclear inclusion bodies formed by WSSV infection were observed in tissues of ectodermal and mesodermal origin of the shrimp such as gills. The other histological changes included nuclear hypertrophy and cellular degeneration. During the early stages of infection (6 h and 24 h) cells showed eosinophilic intra nuclear Cowdry A-type inclusion bodies and at a later stage of 48 h and moribund stage of infection, the tissues showed intra nuclear basophilic inclusion bodies with multifocal necrosis (Fig. 3).

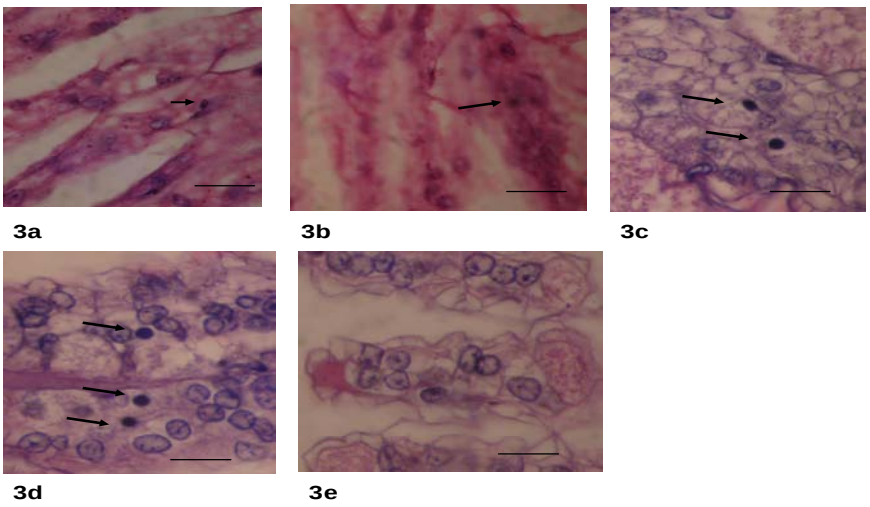

Figure 3. Detection of WSSV in the gill of experimentally infected *P. monodon* at different time intervals by H&E stain (a) 6 h (b) 24 h WSSV infected cells showing eosinophilic intra nuclear inclusion bodies (c) 48 h and (d) moribund stage WSSV infection tissues shows intra nuclear basophilic inclusion bodies. (e) uninfected control gill tissue. Scale bar = 15 µm.

The histological profile of intranuclear inclusions is similar to that reported by others for WSSV in which the inclusions change from eosinophilic to basophilic with age and the nucleus is surrounded by vacant cytoplasm. Progressive increase of inclusion bodies was observed with the increase in duration of infection. The typical histological changes observed in the acute phase of WSSV infection is the presence of prominent eosinophilic to pale basophilic (with H&E stains), Feulgen-positive intranuclear inclusion bodies in hypertrophied nuclei generally of the cuticular epithelial cells and connective tissue cells[14]. Histopathological observations were further confirmed by *in situ* hybridization of gill sections from WSSV infected *P. monodon.*

In situ hybridization has been widely used in general for detection of penaeid shrimp viral disease[1]. In case of WSSV, it has been used to determine tissue distribution of the virus and to study the tissue tropism associated with viral pathogenesis in infected shrimp. Durand et al[5] (1996) reported intense reaction for WSSV in the nuclei located in epithelial cells of the gills and stomach, in the epidermis and in all connective tissues. Positive reaction was observed both in fixed and circulating hemocytes and these were observed throughout the hemocoel and also in the hemal sinuses of the shrimp hepatopancreas. In the present study, the specific probe designed was found to be reactive with the DNA of WSSV inclusion bodies in the tissue sections. Using this DIG labelled 615 bp WSSV VP28 probe *in situ* hybridization revealed positive reactions in the WSSV infected tissues of shrimp. Small numbers of weak positive reactions were observed in the epidermis of the gill tissues in the shrimp samples collected early at 6 h post WSSV infection. The positive hybridization signals gradually increased in the numbers and intensity with progression of disease from 6 h to moribund stage (Fig. 4).

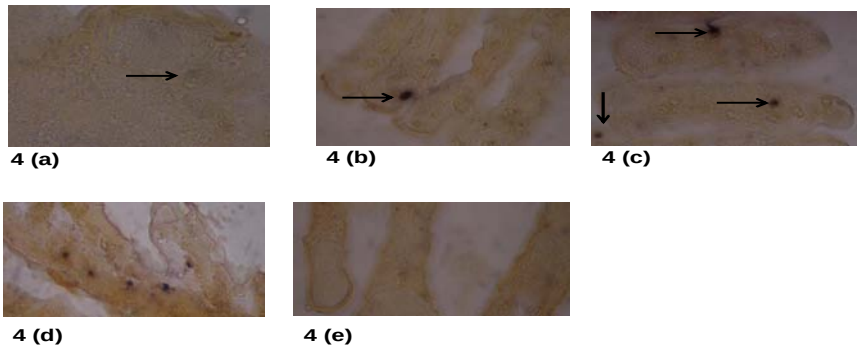

Figure 4. Detection of WSSV in the gill of experimentally infected *P. monodon* at different time intervals by *in situ* hybridization (a) 6 h (b) 24 h WSSV infected cells showing eosinophilic intra nuclear inclusion bodies (c) 48 h (d) moribund stage (e) uninfected control gill tissue. The dark precipitation shows the strong probe reaction. Scale bar = 15 µm.

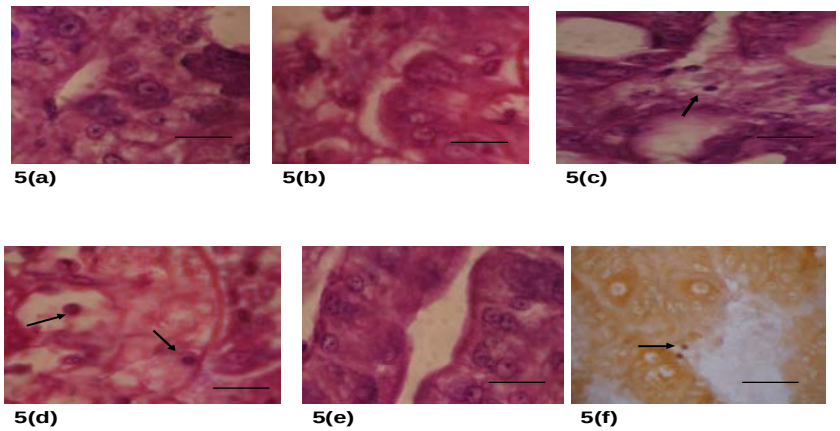

Figure 5. Detection of WSSV in hepatopancreas of experimentally infected *P. monodon* by H&E stain at different time intervals (a) 6 h (b) 24 h (c) 48 h WSSV infected cells showing eosinophilic intra nuclear inclusion bodies (d) moribund stage WSSV infection tissues shows intra nuclear basophilic inclusion bodies (e) uninfected control gill tissue (f) detection of WSSV by *in situ* hybridization at moribund stage of WSSV infection. Scale bar = 15 µm.

Detection of inclusion bodies in hepatopancreas of infected *P. monodon* by H&E stain and by *in situ* hybridization only at later stages of infection (48 h and moribund) indicates that hepatopancreas contains low levels of WSSV infection (Fig. 5). In hepatopancreas, WSSV infection is reported to mainly confine to the myoepithelial cells of the hepatopancreatic sheath and the fibroblasts of the connective tissue. Using *in situ* hybridization it was shown that at late stages of infection most of the cells in the intertubular region were strongly positive and the tubular epithelium remained negative[2]. Epithelial cells of the hepatopancreas are not infected by WSSV, however, positive reaction in some nuclei of the spongy connective tissue of the hepatopancreas has been observed by *in situ* hybridization[5]. The mild histopathological changes observed in infected hepatopancreas is in agreement with results of nested PCR and real time PCR obtained which indicated low levels of WSSV infection in this tissue.

In conclusion, WSSV pathogenesis increased with increase in viral copy numbers in gills which is primary target tissue of WSSV infection. The histopathological and nested PCR results are in agreement with real time PCR data which showed that the virus is able to replicate over time. The combination of techniques used in this study was successful in determining tissue distribution of the virus and the tissue tropism associated with viral pathogenesis in infected shrimp.

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