

Virulence status, viral accommodation and structural protein profiles of white spot syndrome virus isolates in farmed *Penaeus monodon* from the southeast coast of India

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Abstract

The objective of this study was to investigate the reason for variation in the virulence of white spot syndrome virus (WSSV) from different shrimp farms in the Southeast coast of India. Six isolates of WSSV from farms experiencing outbreaks (virulent WSSV; *v*WSSV) and three isolates of WSSV from farms that had infected shrimps but no outbreaks (non-virulent WSSV; *m*WSSV) were collected from different farms in the Southeast coast of India. The sampled animals were all positive for WSSV by first-step PCR. The viral isolates were compared using histopathology, electron microscopy, SDS-PAGE analysis of viral structural proteins, an *in vivo* infectivity experiment and sequence comparison of major structural protein VP28; there were no differences between isolates in these analyses. A significant observation was that the haemolymph protein profile of *m*WSSV-infected shrimps showed three extra polypeptide bands at 41, 33 and 24 kDa that were not found in the haemolymph protein profile of *v*WSSV-infected shrimps. The data obtained in this study suggest that the observed difference in the virulence of WSSV may not be due to any change in the virus, rather it could be due to the shrimp defence system producing certain factors that help it to accommodate the virus without causing any mortality.

Keywords: WSSV, Indian isolates, virulence, haemolymph proteins, viral accommodation

Introduction

White spot syndrome virus (WSSV) is a major viral pathogen affecting shrimp aquaculture globally. It was first reported in 1992 from an outbreak in cultured penaeids in Taiwan (Chen 1992). The virus has a wide host range and affects almost all species of cultured shrimps and crustaceans (Lo, Lin, Ho, Chu, Chen, Yeh & Peng 1997; Rajendran, Vijayan, Santiago & Krol 1999; Sanchez-Martinez, Aguirre-Guzman & Mejia-Ruiz 2007). It is extremely virulent and cumulative mortality can reach up to 100% within 3–7 days post infection.

White spot syndrome virus represents the type species of a new genus of DNA virus Whispovirus, belonging to the family Nimaviridae (Fauquet, Mayo, Maniloff, Desselberger & Ball 2005). It is an enveloped ovoid-shaped virus with a rod-shaped nucleocapsid (Wang, Lo, Li, Chou, Yeh, Chou, Yung, Chang, Su & Kou 1995; Wongteerasupaya, Vickers, Sriurairatna, Nash, Akarajamorn, Boonsaeng, PanYim, Tassanakajon, Withyachumnarnkul & Flegel 1995). The virus contains double-stranded, circular DNA of about 300 kb, which has been completely sequenced in three different WSSV isolates originating from China (WSSV-CN; Yang, He, Lin, Li, Pan, Zhang & Xu 2001), Thailand (WSSV-TH; van Hulten, Witteveldt, Peters, Kloosterboer, Tarchini, Fiers, Sandbrink, Lankhorst & Vlak 2001) and Taiwan (WSSV-TW; GenBank accession no. AF440570). There have been several reports of differences in the virulence of WSSV isolated

from different geographical locations, and these variations have been attributed to deletions in the variable regions of the WSSV genome (Lan, Lu & Xu 2002; Dieu, Marks, Siebenga, Goldbach, Zuidema, Duong & Vlak 2004; Marks, van Duijse, Zuidema, van Hulten & Vlak 2005).

In India, it has been observed that some farmers in Tamil Nadu and Andhra Pradesh (South India) are able to obtain a reasonably good harvest despite the presence of WSSV infection characterized by severe white spots on the carapace. Similar observations have also been made in China (Lan *et al.* 2002). The epizootiological investigations carried out on other viral diseases of *Penaeus monodon* have revealed that the severity of epizootics declined within 1.5–2 years from the first appearance of the new virus (Fegan, Flegel, Sriurairatana & Waiakrutra 1991; Flegel 1997; Owens, Haqshenas, MeElnea & Coelen 1998). These observations suggested the existence of possible genetic variants of WSSV (i.e. 'virulent' and 'non-virulent' strain) by the researchers and farmers. The work reported here, therefore, aimed to compare the putative 'virulent' and 'non-virulent' strains of WSSV and verify whether a strain variation does exist in WSSV in South India. The clinical, light microscopic and electron microscopic characteristics of shrimp infected with these strains are described. Protein profiles of haemolymph of *P. monodon* infected with 'virulent' and 'non-virulent' WSSV are also compared to determine the host response that may be responsible for the resistance of *P. monodon* to WSSV infection.

Materials and methods

Sample source

Shrimp samples were obtained from nine farms located in India (Table 1) during February 2002–December 2002. Of these, six farms experienced white

spot disease outbreak and crop losses. The viral isolates obtained from these farms were tentatively named as 'virulent' WSSV (*v*WSSV). The remaining three farms had successful harvests, although the shrimps were infected with WSSV. The viral isolates obtained from these farms were named as 'non-virulent' WSSV (*nv*WSSV).

Detection of WSSV in shrimp tissues by PCR

DNA was extracted from the gills and pleopods of shrimps. The tissue was homogenized in DNA extraction buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 500 mM NaCl and 1% SDS), boiled for 10 min and then centrifuged at $10\,000 \times g$ for 5 min. The supernatant was precipitated in ethanol and the DNA pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). A two-step PCR amplification was performed using the primer sets reported by Kimura, Yamano, Nakano, Monoyama, Hiraoka and Frousp (1996).

Histopathological study

Tissues (gills and stomach) were preserved in Davidson's alcohol–formalin–acetic acid (AFA) and processed according to the standard procedure of Bell and Lightner (1988). Tissues were sectioned at 4–5 μm thickness and stained with haematoxylin and eosin. The stained sections were observed under an Olympus light microscope (Olympus, Tokyo, Japan) and photomicrographs were taken using a WILD MPS 46 camera (Wetzler, Germany) fitted to a Leitz Laborlux S microscope (Wetzler, Germany).

Electron microscopy

For transmission electron microscopy (TEM) preparation, small pieces (1–2 mm³) of the gills and stomach were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 24 h at 4 °C, followed by three rinses (10 min each) with 0.1 M PBS buffer;

Table 1 Places of collection of different WSSV isolates from the southeast coast of India

Collection location	Host species	Life stages of shrimp	Cultured or wild	'Virulent' or 'non-virulent'
Chidambaram (TN)	<i>P. monodon</i>	Sub-adult	Cultured	'Virulent' (V1)
Marakkanam (TN)	<i>P. monodon</i>	Sub-adult	Cultured	'Virulent' (V2)
Mahabalipuram (TN)	<i>P. monodon</i>	Adult	Cultured	'Non-virulent' (NV1)
Kovalam (TN)	<i>P. monodon</i>	Adult	Cultured	'Non-virulent' (NV2)
Kattur (TN)	<i>P. monodon</i>	Adult	Cultured	'Virulent' (V3)
Gudur (AP)	<i>P. monodon</i>	Sub-adult	Cultured	'Virulent' (V4)
Nellore (AP)	<i>P. monodon</i>	Adult	Cultured	'Virulent' (V5)
Kakinada (AP)	<i>P. monodon</i>	Adult	Cultured	'Virulent' (V6)
Bhimavaram (AP)	<i>P. monodon</i>	Adult	Cultured	'Non-virulent' (NV3)

the specimens were post fixed in buffered osmium tetroxide for 2 h. After rinsing it again in the same buffer, the specimens were dehydrated in graded ethanol and embedded in Epon 812 resin (Merck, Darmstadt, Germany). Both 1- μ m-thick and ultra-thin sections were cut on a Reichert-Jung ultra microtome (Wetzler, Germany) fitted using a diamond knife. The ultra-thin sections were stained with 0.5% uranyl acetate, followed by lead citrate (Reynolds 1963). These sections were examined and photographed under a JEOL-JEM 100SX transmission electron microscope (Jeol, Tokyo, Japan).

Isolation of intact WSSV viral particles

To purify the virus, gill tissues were removed from each of the nine WSSV-infected shrimps. The tissues were homogenized in TNE buffer containing protease inhibitor 1 mM PMSF (phenyl methyl sulphonyl fluoride) and centrifuged at $3000 \times g$ for 5 min at 4 °C. The supernatant was centrifuged at $100\,000 \times g$ for 1 h at 4 °C (Beckman Coulter ultracentrifuge, SW41 rotor, Beckman Coulter, CA, USA). Then, the pellet was resuspended in 1 mL of TNE buffer. The suspension was overlaid on the top of a 10–50% (w/v) continuous sucrose gradient and centrifuged at $123\,000 \times g$ for 90 min at 4 °C. The viral band was collected and the fraction was diluted in the ratio 1:10 using TNE buffer and centrifuged at $123\,000 \times g$ for 1 h. The pellet was then resuspended in 100 μ L of TNE buffer and stored at –70 °C until further use. The degree of purity of virus isolated was evaluated by negative-staining TEM. For TEM examination, each virus suspension was mounted on a carbon-coated nickel grid (400 μ m mesh) and negatively stained with 2% phosphotungstic acid, and the specimens were examined under a Hitachi H600 transmission electron microscope (Hitachi, Tokyo, Japan).

SDS-PAGE analysis of purified WSSV virions

Purified intact WSSV virions were analysed by SDS-PAGE. The total protein was estimated using the standard method of Lowry, Rosebrough, Farr and Randall (1951). About 30–50 μ g of protein was separated by 12% SDS-PAGE and visualized using Coomassie Brilliant Blue R-250 staining.

Haemolymph protein profile

The haemolymph was collected from infected shrimps (both *v*WSSV and *n*WSSV) under sterile conditions. The haemolymph was allowed to clot, and the serum

was separated by centrifugation at $3000 \times g$ for 5 min. The serum was transferred to a fresh tube and the sample was separated by 10% SDS-PAGE.

Cloning, sequencing and computer analysis of viral envelope protein VP28

The vp28 gene of WSSV is of considerable significance in WSSV pathogenicity, among the other important viral proteins. Subunit vaccines (Witteveldt, Cifuentes, Vlask & van Hulten 2004; Witteveldt, Vlask & van Hulten 2004) and dsRNA and siRNA targeting this gene (Robalino, Bartlett, Shepard, Prior, Jaramillo, Scura, Chapman, Gross, Browdy & Warr 2005; Westenberg, Heinhuis, Zuidema & Vlask 2005) have been shown to confer significant protection in shrimp against WSSV. To determine whether any mutational changes in this gene could contribute to the observed difference in virulence, vp28 was amplified from each of the 'virulent' and 'non-virulent' isolates using gene-specific primer designed from the vp28 sequence in GenBank (GenBank accession no. DQ007315). The PCR primers used were forward primer 5'-AGAGA ATTTCATGGATCTTTCTTTTCAC-3' (*Eco*RI site in italics) and reverse primer 5'-CACGTCGACTTACTCGGTCTC AGTGC-3' (*Sal*I site in italics). PCR was carried out using the following profile: 5 min at 95 °C, 30 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 5 min. The amplified product was digested and cloned into the plasmid vector pET20b (+) (Novagen, Darmstadt, Germany). One of the clones obtained was sequenced. Sequencing was carried out on one end of the cloned fragment using the universal T7 terminator primer. The sequencing was performed by a commercial sequencing company (Microsynth, Switzerland). The deduced amino acid sequence was analysed for homology to other proteins contained in the public database Genbank (BLASTP). The amino acid sequences of vp28 obtained in the present study and other published sequences of vp28 from GenBank: Vietnam (accession no. CAD83839), the Netherlands (accession no. AAF29807), Korea (accession no. AAP87278), Japan (accession no. AAP06670), Indonesia (accession no. AAP06668), China (accession no. AAY27882) and the United States of America (accession no. AAP06661) were subjected to multiple alignments using CLUSTAL W (1.82).

Shrimp culture

Healthy *P. monodon* postlarvae (PL) (15–20 days old), belonging to an individual broodstock that tested

negative for WSSV and MBV, were obtained from a commercial shrimp hatchery in Chennai, India. Representative animals were again screened for WSSV using the PCR method described in 'Detection of WSSV in shrimp tissues by PCR' and for MBV using a standard squash mount preparation described by Lightner, Redman and Bell (1983). After confirming the WSSV- and MBV-free status, the larvae were reared in a 2000-L concrete tank (salinity 22 g L^{-1} , temperature $27\text{--}29^\circ\text{C}$) on a commercial diet in the laboratory (CIBA, Chennai, India) until the animals gained 5–6 g body weight.

In vivo shrimp infectivity test

An inoculum of WSSV was prepared from the gills of both *v*WSSV- and *m*WSSV-infected shrimps. From each shrimp sample, 100 mg tissue was homogenized in 1 mL TNE buffer (50 mM Tris-HCl, 400 mM NaCl, 10 mM EDTA, pH 7.4) and centrifuged at $1500 \times g$ for 10 min at 4°C . The supernatant was filtered through a $0.45 \mu\text{m}$ filter (Sartorius, Germany), and the filtered suspension was diluted in the ratio of 1:50 with 0.9% NaCl.

A total of 300 juveniles ($5.5 \pm 0.5 \text{ g}$) were used for the infectivity tests. The animals were divided into 30 groups, comprising 27 test groups and three control groups, with three replicates for each WSSV isolate, and stocked in 10-L aquaria ($10 \text{ animals tank}^{-1}$). Each shrimp from the test group was injected with $50 \mu\text{L}$ WSSV inoculum at a point between the second and the third tergal plates on the lateral side. The control groups were injected with extracts from the gills of WSSV-negative *P. monodon* prepared in the same manner as described for the test groups.

Results

Gross morphology

Gross morphology of shrimps infected with *v*WSSV and *m*WSSV showed typical clinical signs of WSSV infection. Virulent WSSV-infected shrimps showed white spots on the carapace, reddish discoloration on the body surface and appendages, lethargy, loss of balance, reduced feeding and they finally died. No clinical signs, other than apparent white spots on the carapace, were observed on the shrimps infected with *m*WSSV. Furthermore, these animals were found to be healthy with normal feed intake and survived until harvest.

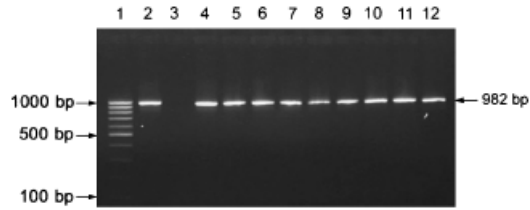


Figure 1 Detection of WSSV in the shrimp samples collected at different places. All samples were positive by first-step PCR. Lane 1: 100 bp DNA ladder, lane 2: positive control, lane 3: negative control, lanes 4–12: shrimp tissue samples in order of collection site (please see Table 1 for order).

Polymerase chain reaction (PCR)

In PCR amplification, all the nine isolates were found to be first-step PCR positive for WSSV (Fig. 1). A band corresponding to 982 bp was detected after electrophoresis of the PCR products of all the nine isolates.

Histopathology

Histopathological changes in the tissues of *v*WSSV-infected (Fig. 2a) and *m*WSSV-infected (Fig. 2b) shrimps were similar. These changes were characterized by nuclear hypertrophy, chromatin margination, variable multifocal necrosis and haemocyte encapsulations. In *m*WSSV-infected shrimps, histopathological changes were less severe and widespread cellular degeneration, as noticed in *v*WSSV-infected shrimps, was not observed.

Transmission electron microscopy

Transmission electron microscopic observations of *v*WSSV- (Fig. 3a) and *m*WSSV-infected (Fig. 3b) tissues showed similarity in size and morphology. The size of the larger virion ranged between $112 \text{ and } 268 \pm 34 \text{ nm}$ in length, whereas the smaller virion ranged between $98 \text{ and } 260 \pm 30 \text{ nm}$ in length. The size of the nucleocapsid varied from $82 \text{ to } 246 \pm 35 \text{ nm}$. A paracrystalline array of virus was observed within the nucleus. The virion is typically characterized by an apical envelope extension. Rod-shaped to elliptical virus particles surrounded by a trilaminar envelope were found in the nuclei of affected cells.

Isolation of WSSV

After sucrose gradient centrifugation, a white band thought to contain the purified virus was observed in the middle of the gradient. The viral band was

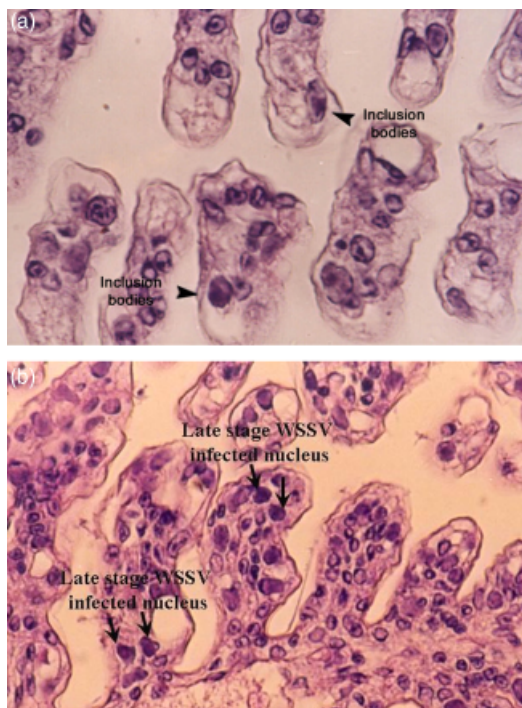


Figure 2 (a) Light photomicrograph of histological section through a region of non-virulent WSSV-infected gills of *Penaeus monodon*. Cells showing eosinophilic intracellular inclusions (arrow heads) surrounded by margined basophilic chromatin are observed (b) Histological section through a region of virulent WSSV-infected gills of *P. monodon*. There is cellular degeneration with nuclear hypertrophy. Late stage WSSV-infected nuclei are seen.

collected. The purity of the virions was determined by TEM. The shape of the negatively stained intact WSSV virions was rod-shaped to elliptical. Naked viral nucleocapsid cores were observed (Fig. 4a), and each intact virion had a long tail-like extension at one end (Fig. 4b). Both ν WSSV and n WSSV virions were alike.

Viral structural protein profile

More than 20 bands of different intensities were observed in all the groups on staining with Coomassie Brilliant Blue R-250. The protein profile of all the nine isolates was identical (Fig. 5). Eight major bands at 75, 69, 34, 27.5, 24, 18, 13.5 and 11 kDa were clearly observed. Of these, the 75 and 69 kDa bands corresponded to shrimp haemolymph proteins that were co-purified with the virus (van Hulst, Westenberg, Goodall & Vlak 2000). The protein profiles obtained

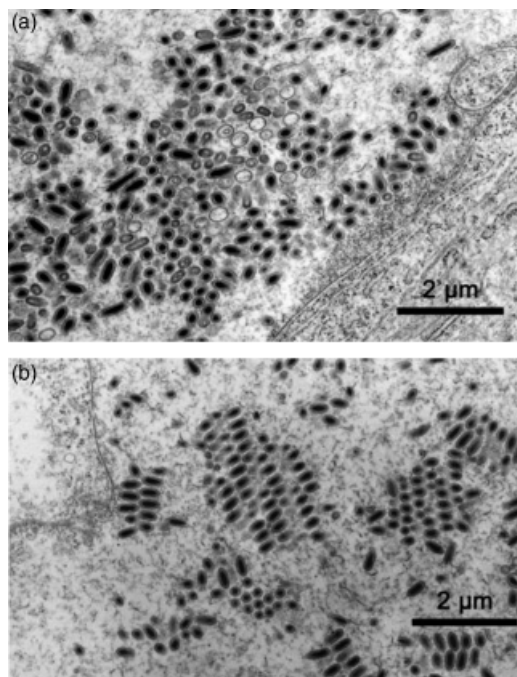


Figure 3 Transmission electron micrograph of ultra-thin section of WSSV-infected stomach epithelial cells of *Penaeus monodon*. (a) ν WSSV-infected shrimp tissue section. (b) n WSSV-infected shrimp tissue section. Note the arrangement of viral particles in paracrystalline array.

from the ν WSSV and n WSSV were similar, and no variation was observed between the two.

Haemolymph protein profile

We found three additional major bands corresponding to 41, 33 and 24 kDa in the haemolymph of n WSSV-infected shrimp (Fig. 6), which were not present in the haemolymph protein of shrimp affected with ν WSSV. These additional bands were not found in any of the other lanes. The other protein bands were similar in virulent- and non-virulent virus-infected shrimp. Haemolymph protein profiles from control shrimp were comparable, except for the three additional proteins detected in n WSSV shrimp protein.

Sequencing and comparison of VP28

The viral coat protein VP28 is a major structural protein of WSSV (van Hulst *et al.* 2000) and has been implicated in the systemic infection of shrimp by

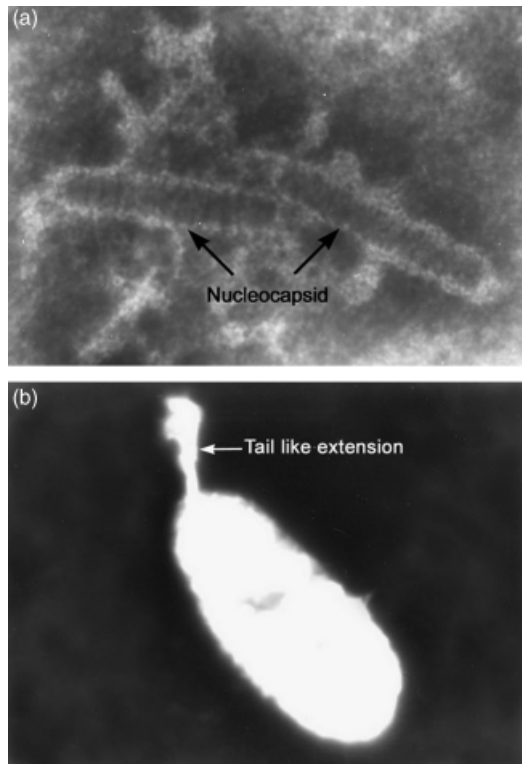


Figure 4 Electron micrograph of negatively stained WSSV purified by sucrose gradient. (a) Enveloped rod-shaped nucleocapsid is clearly observed. There is a distinct vertical helix located horizontally along the axis of the nucleocapsid core. (b) Complete virion with the tail-like extension is observed.

WSSV (van Hulten, Witteveldt, Snippe & Vlak 2001; Yi, Wang, Qi, Yao, Qian & Hu 2004). In order to determine whether there were any differences in the gene sequence of *vp28* of these two isolates, the gene was amplified from one *v*WSSV and one *n*WSSV isolate and sequenced. The *vp28* sequences of the 'virulent' and 'non-virulent' isolates were 100% identical to each other. Thus, there was no difference in the *vp28* sequences of 'virulent' and 'non-virulent' isolates. When compared with the sequences in GenBank, our sequence showed 100% similarity to those obtained in Vietnam, the Netherlands, Korea, Japan, Indonesia, China and the USA. The deduced amino acid sequence of *vp28* in this paper and the previously published sequences were aligned using CLUSTALW (1.82) multiple sequence alignment (EMBL-EBI). All the sequences showed 100% homology, except the USA isolate, which differed at amino acid 40, where histidine is replaced by arginine (data not shown).

In vivo infectivity studies of the nine WSSV isolates

To understand the difference in the pathogenicity of the two types of virus isolates, challenging studies were undertaken to determine whether the difference in infectivity could be attributed to changes in the virus itself. Challenging the *v*WSSV and *n*WSSV isolates to juvenile *P. monodon* showed that all the nine isolates were highly pathogenic to the animals. The cumulative mortality reached 100% within 5 days post infection in all the test groups. No mortality was observed in the control groups. White spot syndrome virus infection of all moribund shrimps in the test groups was confirmed by WSSV-nested PCR, and all the infected animals were found to be first-step PCR positive. The control groups were negative for WSSV by second-step PCR.

Discussion

In the study presented here, the possible existence of a 'virulent' and a 'non-virulent' WSSV was analysed using histopathological, electron microscopical and molecular data.

Histopathological observation showed that both *v*WSSV- and *n*WSSV-infected tissues had symptoms of WSSV infection, as reported earlier (Rodriguez, Bayot, Amano, Panchana, de Blas, Alday & Calderon 2003), although there was a difference in the severity of symptoms. The histopathological changes in the *v*WSSV-infected tissues were markedly severe, which is to be expected because the shrimps were collected during the outbreak time and many animals had died in these farms due to the disease. Study of the WSSV latency-related gene in specific pathogen-free (SPF) shrimp (Khadijah, Neo, Hossain, Miller, Mathavan & Kwang 2003) reported the presence of WSSV in a latent state in the SPF stock. In this study, however, it cannot be argued that the virus in *n*WSSV-infected shrimps was in a latent state because the animals tested positive for WSSV by first-step PCR showed obvious signs of infection such as white spots on the exoskeleton and typical intranuclear inclusions of WSSV in the affected tissues. Electron microscopic observation of the infected tissue sections and purified *v*WSSV and *n*WSSV virions showed similar morphologies, with no discernible difference in size or shape.

In the present study, we found that both *v*WSSV and *n*WSSV isolates caused similar mortality patterns in *P. monodon*, with 100% mortality caused by

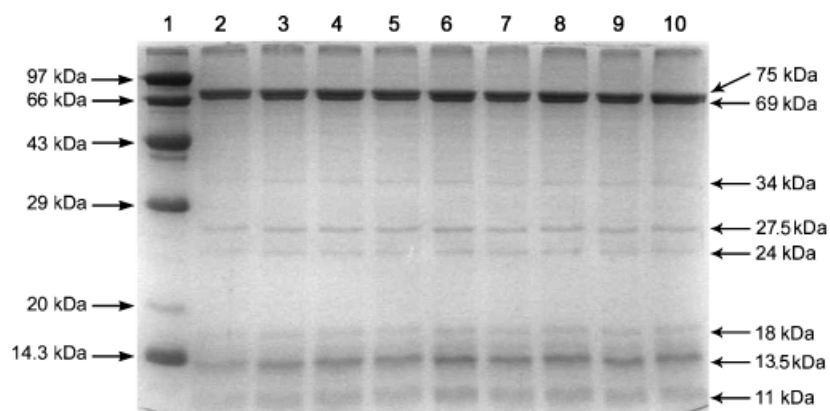


Figure 5 SDS-PAGE protein profile of the nine WSSV isolates. Lane 1: protein molecular weight marker; Lanes 2, 3, 6, 7, 8 and 9: V1, V2, V3, V4, V5 and V6 respectively; Lanes 4, 5 and 10: NV1, NV2 and NV3, respectively, where V, virulent isolate and NV, non-virulent isolate (see Table 1 for details). Eight major bands corresponding to 75, 69, 34, 27.5, 24, 18, 13.5 and 11 kDa were clearly observed. Of these, the 75 and 69 kDa bands correspond to shrimp haemolymph proteins.

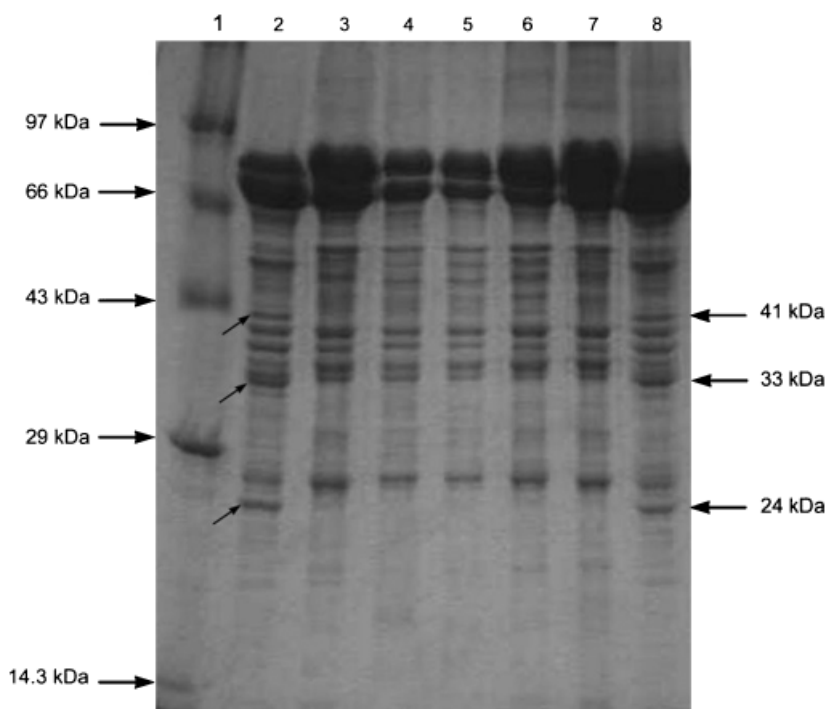


Figure 6 SDS-PAGE profile of shrimp haemolymph from vWSSV- and mWSSV-infected animals. Lane 1: medium range protein molecular weight marker, Lanes 2 and 8: haemolymph of shrimp infected by mWSSV, Lanes 3–7: haemolymph of shrimps infected by vWSSV. The three additional protein bands obtained in the haemolymph of animals infected with mWSSV are indicated by arrows.

both isolates within 5 days. This is in marked contrast to the finding of Marks *et al.* (2005), wherein there was a significant difference in the median lethal time of the less virulent TH-96-II isolate (14 days) and the more virulent WSSV-TH isolate (3–5 days). The authors had suggested that WSSV-TH, which has a smaller genome (~ 293 kb), may pos-

sess a replication advantage when compared with the TH-96-II isolate, which has a larger genome (~ 312 kb), and this could be the reason for its higher virulence.

Analysis of the viral protein profile of the two isolates showed identical profiles with no discernible difference. Mutational changes in amino acids of ma-

major viral proteins that play an important role in infectivity have been shown to cause a change in the pathogenicity of the virus. Recently, a new variant of the Taura syndrome virus (TSV) called the Belize isolate has been described that varies in virulence when compared with the Hawaii isolate and was found to belong to a distinct group on performing phylogenetic analysis of a major capsid protein-encoding gene (Tang & Lightner 2005). It has been reported that a single amino acid change in the E2 spike protein of a virulent strain of Semliki Forest virus, which is lethal to mice, attenuates pathogenicity and causes the virus to become avirulent when given to adult mice (Glasgow, Killen, Liljestrom, Sheahan & Atkins 1994). Several WSSV viral proteins have been implicated in the infectivity in shrimp including VP28, VP31, VP36B, VP68, VP76, VP281 and VP466 (van Hulten, Witteveldt, Snippe *et al.* 2001; Huang, Xie, Zhang & Shi 2005; Li, Xie & Yang 2005; Wu, Wang & Zhang 2005; Li, Yuan, Cai, Gu & Shi 2006). To understand whether a mutation in the genes encoding these proteins could contribute to the difference in virulence observed in this study, we amplified and sequenced the *vp28* gene from both the isolates. *vp28* encodes a major structural protein of WSSV that has been implicated in the systemic infection of shrimp (van Hulten, Witteveldt, Snippe *et al.* 2001) and has been shown to be involved in the binding and entry of WSSV into shrimp cells by an *in vitro* binding assay (Yi *et al.* 2004). The sequence of *vp28* from both our isolates showed 100% similarity, ruling out any mutations in the *vp28* amino acid composition. However, it is necessary to examine all WSSV proteins involved in the infectivity process to rule out the possibility of any mutations in the genetic make-up of the isolates.

On analysing haemolymph protein profile, it was found that the haemolymph of shrimp infected with the *m*WSSV isolates revealed three bands corresponding to 41, 33 and 24 kDa, which were not observed in any of the other lanes. These bands may correspond to some protein expressed only by shrimp that have developed some resistance or tolerance to WSSV. This could explain why the same isolate could produce rapid mortality upon experimental infection in the laboratory. The experimental animals were raised from the PL stage under closed laboratory conditions without any exposure to infectious agents and cannot develop any tolerance to the viral infection. Substances with broad anti-viral activity in tissues of crustaceans have been reported (Pan, Kurosky, Xu, Chopra, Coppenhaver, Singh & Baron 2000). An earlier publication has reported the anti-

viral nature of an approximately 28 kDa protein called *PmAV* in *P. monodon* and has suggested that this protein may play an important role in the defensive mechanism of *P. monodon* against viruses (Luo, Zhang, Shao & Xu 2003). In another report, the resistance against WSSV observed in 'immune' kuruma shrimp was attributed to the virus-neutralizing factor(s) in the haemolymph, which appeared 3 weeks after exposure to the virus and lasted for about 1 month (Wu, Nishioka, Mori, Nishizawa & Muroga 2002). In a recent report, two anti-viral factors were identified in haemocytes of WSSV-resistant *Penaeus japonicus*, of which one was an interferon-like protein (InHP) and the other was a (2'-5') oligo(A) synthetase-like protein (He, Qin & Xu 2005).

An earlier report had proposed a new concept of 'active viral accommodation' for crustacean response to viral pathogens (Flegel & Pasharawipas 1998). This concept was proposed to explain the lack of a tissue response or apoptosis in response to viral pathogens in crustaceans that enables the host to tolerate viral infection without mortality. It further proposed that accommodation is characterized by the absence of active defence against the viral pathogen. It was proposed that shrimp had a specific 'recognition mechanism' by which they could acquire 'tolerance' to the new viral pathogen during their larval development. By accommodating viruses in persistent infections without mortality, there would be positive selection of viral variants with the least negative effect on the host (Flegel 2007). The present study finds resemblance to the concept of 'active viral accommodation' proposed by Flegel and Pasharawipas (1998). Furthermore, it is also possible that the '*m*WSSV-infected shrimps' could have encountered WSSV in an innocuous form early in their larval stages during stocking in the ponds, which may have given them some tolerance to the virus. In this context, it is proposed that the three extra protein bands observed in the haemolymph protein profile of *m*WSSV-infected shrimp may be a similar anti-viral protein or may be some factor(s) associated with resistance or tolerance to WSSV.

Considerable progress has been made in the characterization of WSSV, but the understanding of shrimp's defence system in response to viral infection is still poor. It is essential to consider the virus-host interaction while studying the change in virulence of WSSV rather than looking at the virus or shrimp alone. Such a holistic approach can further our understanding of shrimp response and adaptation to

viruses and may, in the long run, help us to find new treatment methods for viral diseases in shrimp.

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