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## DNA based diagnosis of fish / shellfish pathogens

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Diagnosis forms the first step in any disease control programme, which determines the ultimate success or failure of the programme. The diagnostic procedure may include a single diagnostic test or a battery of tests. However, the diagnosis can often get complicated in the cases of mixed infections, with the involvement of primary, secondary and even tertiary pathogens. The different types of diagnostic methods used in aquaculture include microscopy, histology and histopathology, electron microscopy, culture and biochemical identification, bioassay, serological methods, tissue culture and molecular diagnostics.

**Light microscopy:** Bright-field, phase contrast or dark field microscopic observation on wet mounts and stained or unstained tissue of abnormal or diseased animals.

- Required resource: Experienced laboratory technicians.
- Nature of diagnosis: Primary, inconclusive.

**Histology and histopatholgy:** Routine histological and histochemical examination of tissue sections.

- Required resource: Laboratory facilities and experienced laboratory technicians.
- Nature of diagnosis: Secondary. Method provides specific information but poor in sensitivity and speed.

**Electron microscopy:** Ultrastructural examination of infected tissue sections, negatively stained virus preparations or surface scanning of samples.

- Required resource: Expensive laboratories and expertise.
- Nature of diagnosis: Conclusive, but is time consuming and laborious.

**Culture and biochemical identification:** Standard culture methods of bacteria and fungi using selected artificial media preparations followed by biochemical tests.

- Required resource: Good laboratories and expertise.
- Nature of diagnosis: Conclusive. But is slow, and time consuming

**Bioassay:** Laboratory challenge of the candidate species with selected pathogen.

- Required resource: Wet laboratory and expertise.
- Nature of diagnosis: conclusive, but slow and time consuming.

**Serological Methods:** Use of specific antibodies as diagnostic reagents in immunoblot, agglutination, diffusion, hybridisation etc.

- Required resource: Good laboratories and expertise
- Nature of diagnosis: Conclusive, different levels of sensitivity.

**Tissue culture:** *In vitro* culture of pathogens in tissue culture systems, or in primary cell cultures.

- Required resource: Sophisticated laboratories with expertise.
- Nature of diagnosis: Conclusive but expensive and time consuming.

**Molecular diagnostics:** Amplification and detection of unique sections of pathogen' s genome.

- Required resource: Sophisticated laboratories and expertise.
- Nature of diagnosis: Rapid, specific, most sensitive, and conclusive.

Conventional diagnostic procedures listed above have their own limitation especially in speed, specificity and sensitivity. Many molecular techniques are potentially faster or more sensitive than methods such as culture, serology and histology that are traditionally used to identify fish diseases. In addition, the application of molecular tools enables the detection of genetic variations that denote subspecies or strains. A comparison of the above diagnostic techniques shows that each one has its own advantages and disadvantages. But when we consider the specificity and sensitivity of the tests, molecular tools have a definite edge over the others. DNA based new generation diagnostic tools such as PCR has emerged as the solution for rapid diagnosis with speed, specificity and sensitivity. Techniques of major significance include polymerase chain reaction (PCR) amplification of nucleic acids, restriction enzyme digestion, probe hybridisation and nucleotide sequencing. An added advantage of these molecular diagnostic techniques is besides identifying the pathogen species, it has the ability to discriminate below the level of species and identify strains.

**Polymerase chain reaction (PCR) and Screening of seed and broodstock/ spawners :** In the early asymptomatic stages of viral infections, conventional diagnostic tools fail to detect the carrier hosts. Virus being the smart pathogen with its ultra size, host and tissue preference, reproduction efficiency and lethality, is able to evade the detection levels of conventional diagnostic tools. This is where the DNA based diagnostic technologies emerged as new generation diagnostic tool, which has all the features of a smart diagnostic tool with all the three 'S', Sensitivity, Specificity and Speed, to detect the viral pathogens. Among the DNA-based diagnostic technologies, polymerase chain reaction (PCR) is at the forefront of molecular diagnostic technology toady. PCR technique has been proven to be the most sensitive and specific diagnostic tool for the screening of WSSV in shrimp seeds and mother prawns. Within a couple of hours, PCR technique can amplify from even a single DNA molecule of the pathogen to millions of copies, which can be easily detected using techniques such as electrophoresis.

In India, viral diseases have not been considered to be a significant factor in marine and brackish water finfish culture, but there have been many reports indicating the existence of viral diseases which cause severe mortalities in fin fishes worldwide. Viral encephalopathy and retinopathy (VER), or viral nervous necrosis (VNN) has been reported as a serious disease of larval, juvenile and sometimes in older marine fish that occurs almost world-wide. It is known to cause the disease in groupers, pleuronectids, snappers, white bass, sea bream, Atlantic halibut, large mouth bass and

freshwater aquarium fishes. One of the important sources of the nodavirus is the carrier fish that neither show clinical symptoms nor die due to the VNN. Diagnosis is based on examination of histological sections of brain and retina and by RT-PCR of these organs.

**Viral diseases and PCR diagnosis in India :** PCR techniques was established as a diagnostic tool in the early nineties. Since 1997, PCR has been in use by the Indian shrimp farmers for the screening of *Penaeus monodon* seeds produced by the hatcheries for WSSV, through the PCR laboratories established by the private promoters, who have used the nested PCR procedures developed in Taiwan and Thailand. Later, institutions such as Mangalore Fisheries college-Mangalore, Central Institute of Brackishwater Aquaculture (CIBA)-Chennai and CMFRI, Cochin, developed the nested PCR for the detection of WSSV in India. PCR labs established by Marine Products Development Authority (MPEDA) at various farming centers further popularized the PCR among the farmers.

Efforts initiated by MPEDA and CIBA with NACA has developed benchmark for PCR harmonization in India with special to the screening of WSSV. Presently, most of the government institutions in India, engaged in the shrimp diseases are equipped with PCR diagnostic facilities, and most of these facilities are available for the use of shrimp farmers. CIBA is planning to set up a referral PCR laboratory at it head quarters at Chennai, to solve the intra and inter laboratory conflicts in PCR results.

**Practical issues with PCR detection :** Presently, there is no common standard in the PCR diagnosis in the country, among the various PCR laboratories and Government institutions in India. Lack of standardized methodologies can produce inconsistent results which can hamper the reliability and comparability of the diagnosis, and further it will affect the decision making process in health management. The use of PCR technique for health management necessitates a higher level of validity because of the therapeutic and management decisions rests on the outcome of the test. Areas that require standardization are;

**Sample collection :** Collection of samples for PCR analysis and the method of preservation should be simple and practical. It may not be always possible to bring live or frozen sample to the PCR lab for analysis. Tissue samples or whole larvae for PCR can be preserved in 95% ethanol. Ethanol fixed samples are easy to handle, and the sample can be sent to the PCR labs by courier. Ethanol preserved samples were routinely used for the PCR at CIBA, and the results were always good and comparable to the live/frozen samples.

**Tissues used for PCR :** The proper selection of DNA sources is critical in obtaining accurate PCR results. Though all the shrimp tissues of ectodermal and mesodermal origin harbours WSSV, tissue has to be selected on the basis of their PCR amplification and consistency. In our laboratory, DNA template from the tissues such as gill, epidermis, stomach wall, eyestalk (without compound eye) and pleopod gave good and consistent PCR amplification. However, the gill tissue or pleopod is the preferred one. Eyestalks, when used along with the compound eyes, PCR inhibition was observed as reported by Lo *et al.* (1998). When postlarvae were used for PCR amplification, their compound eyes were removed to prevent any PCR inhibition.

**DNA extraction procedure :** Time consuming DNA extraction procedure cannot be suggested in rapid diagnostics. Instead, simple and time saving extraction procedure to produce quality DNA

template has to be used. The extraction procedure should minimize the use of PCR inhibitory substances viz. Phenol, SDS, etc. A simple, rapid and cost-effective DNA extraction procedure developed at CIBA using alkaline lysis coupled with boiling provided good quality DNA for PCR, in just 15 minutes. The DNA quality was comparable to the DNA extracted using standard methods.

**DNA template :** The amount of template DNA required for a successful PCR amplification has to be standardized with respect to the size and tissue of the shrimp. An excess quantity of DNA template can inhibit the PCR reaction, causing false negative results. When the samples are pooled for PCR analysis, adjustments have to be made in the sample size and DNA-template concentration to avoid possibility of PCR inhibition due to excess DNA.

**PCR and risk of contamination :** Extreme sensitivity of PCR, can directly lead to the greater risk of contamination. PCR facilities should include separate sample preparation room and amplification room. PCR lab must be clean, and strict discipline should be followed in handling dedicated micropipettes and disposable tips. To prevent cross contamination between samples, disposable tissue homogenizers, centrifuge tubes, and pipette tips have to be used during the DNA-template preparation. Use of low contamination strategies by developing users' friendly PCR kits is required. Positive and negative controls should be included in each reaction to evaluate any false positive/ negative results.

Once a common standard is adopted, the reproducibility and reliability of PCR diagnosis among different laboratories will be high. The PCR data generated among these labs can be used for epidemiological studies in the region, which will be useful in developing the health management strategies for shrimp farming.

**PCR and challenges on shrimp health management in India**

**PCR screening and the hatchery factor :** The best expected and useful application of PCR in shrimp farming is its use as a health management tool in the screening of broodstock/spawners, against disease causing pathogens. In the right health management approach, one would expect the PCR testing of the broodstock/spawners and subsequent rejection or acceptance of the mother shrimp in the hatchery, according to the test status. However, this is not happening in the Indian hatcheries. Majority of the hatchery operators use spawners, irrespective of the PCR results. One of the reasons for this attitude is the high cost involved in the purchase of the spawners (Rs. 3000 to Rs. 50,000). However, the high investment in the 'quality broodstock' incurred by the hatchery operators by discarding the infected ones can be recovered by selling 'WSSV free premium shrimp seeds' for high price. Unless and until the hatchery operators adopt the proper (truthful) screening of spawners on the basis of PCR, the testing of seed from the farmers end is just an exercise, without any binding on the real pathogen status on the seeds.

**Threat of horizontal virus infection :** Water sources such as canals, creeks and estuaries for the shrimp farms in farming areas are already contaminated with WSSV, the purpose of PCR screening of shrimp seed can become simply futile. Further, animals (e.g. dogs and foxes) and common birds (e.g. crows) which freely move in the farming areas, can act as potential agents in horizontal transmission of WSSV.

The purpose of PCR screening can be fruitful, only when the dual possibilities of vertical and horizontal transmission of the virus, mentioned above is prevented. One can imagine the wishful

thinking of using SPF seed and its ultimate fate in the context of potential horizontal transmission of WSSV!

**The communication factor :** Unlike other animal rearing systems, shrimp farming is a complex and multi faceted activity where so many factors are converging in the three dimensional environment of the culture facility. Hence when a technology such as PCR is applied, there should be a perfect harmony and communication between the hatchery operators, farmers, processors, traders, scientist, social workers and the policy makers. Unfortunately, in India, the communication channels among the above are poor, and many are working in isolated compartments. This will undermine the application of technologies, producing mostly negative results. Formation of an 'Indian shrimp farming consortium (IFC)' linking all the stake holders in Indian shrimp farming, in a level playing field may be a solution, to establish the effective communication, for the progress of shrimp farming.

**Change in virulence/viral accommodation :** Differential virulence and mortality have become common among the shrimp farms infected with WSSV. Nature of WSSV outbreak and mortality pattern has become most unpredictable and health management effort has become arbitrary. In spite of fully blown up white spot disease, instances are many where farmers were able to harvest fully grown marketable shrimp of 30-40 grams. This can be due to a decreased WSSV virulence level due to viral mutation, or due to an increased tolerance for the virus by the shrimps. Existence of different WSSV strains with pathogenic and non-pathogenic nature is a possibility. Now that the whole WSSV genome is sequenced, development of PCR diagnosis to differentiate pathogenic form from non-pathogenic variant can be a new diagnostic challenge.

**Limitations**: However, molecular diagnostic techniques have some limitations in terms of appropriate applications, standardized sampling, testing procedures and interpretation of results and are also of limited value to newly emerging diseases where the causative agent is unknown.

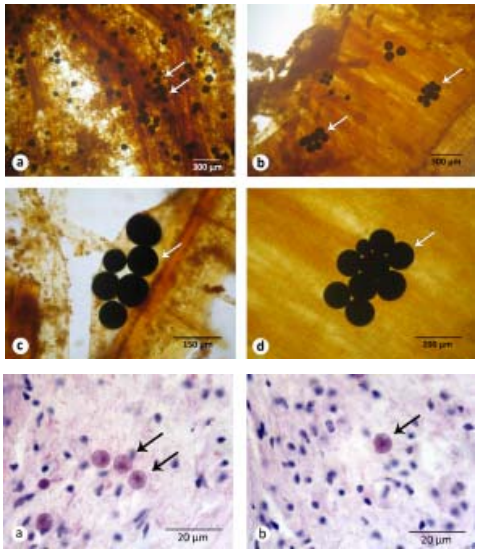
In the case of new and emerging pathogens, conventional methods such as clinical symptoms, light microscopy, histopathology and electron microscopy is required to diagnose the pathogen and purify the pathogen so as to generate genetic details such as sequence information required for molecular diagnosis.

**Case study 1 :**

**Diagnosis of the protozoan parasite *Perkinsus olseni* infection in *Pinctada fucata*.**

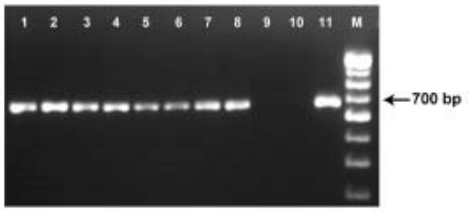
The pearl oyster, *Pinctada fucata* (Gould), is a commercially important bivalve distributed in the Gulf of Mannar along the southeast coast of India. During the past few decades, the natural pearl oyster beds in the Gulf of Mannar have showed a sharp decline leading to the closure of the traditional pearl fishery and was presumed to be due to over-exploitation and pollution.

The pathogen profile of *P. fucata* from the southeast coast of India has not been studied at all. Since *Perkinsus* spp. is known to have destroyed many oyster beds worldwide, the present study was taken up to screen the pearl oyster population along the Gulf of Mannar coast. Samples of *P. fucata* were collected from wild populations at three different locations in the Gulf of Mannar and analysed using Ray's fluid thioglycollate medium (RFTM) culture, histology and molecular diagnostic techniques (PCR).



The current major limitations for identifying the various species of *Perkinsus* are the broad host range encountered and the absence of significant morphological differences among the known species. The standard diagnostic method for *Perkinsus* spp. as per OIE stabdards is the incubation of suspected host tissues in Ray's fluid thioglycollate medium (RFTM) followed by staining with Lugol's iodine. The parasitic stages if present can be visualized as blue-black spheres under light microscope. Though simple and sensitive, it cannot distinguish between various species of *Perkinsus* and some times certain dinoflagellates can give false positive results.

In the present case, Ray's fluid thioglycollate medium assay of the tissues showed enlarged blue-black hypnospores characteristic of *Perkinsus*-like organisms. All the samples collected during the period were found positive showing a prevalence of 100%. But these results cannot be treated as confirmatory. *Perkinsus*-like organisms measuring 4.7 µm to 7.3 µm were observed in the histological preparations. However, typical 'signet ring' stages of trophozoites, a characteristic 'signature' of *Perkinsus* were not observed in the histological preparations studied, again creating some confusion.




The tissues were then screened using the *Perkinsus* genus specific ITS 85 & ITS 750 primers which amplified the product specific to *Perkinsus* sp. (ca. 700 bp) confirming the presence of *Perkinsus* sp. infection beyond any doubt. But again the species level identity of the pathogen was not known. In the next step the PCR products were purified and sequenced and analysed using BLAST. The results showed 99% identity to *P. olseni* with 100% query coverage. The PCR results were in perfect agreement with that of the RFTM and histology studies and confirm the presence of *Perkinsus* spp.

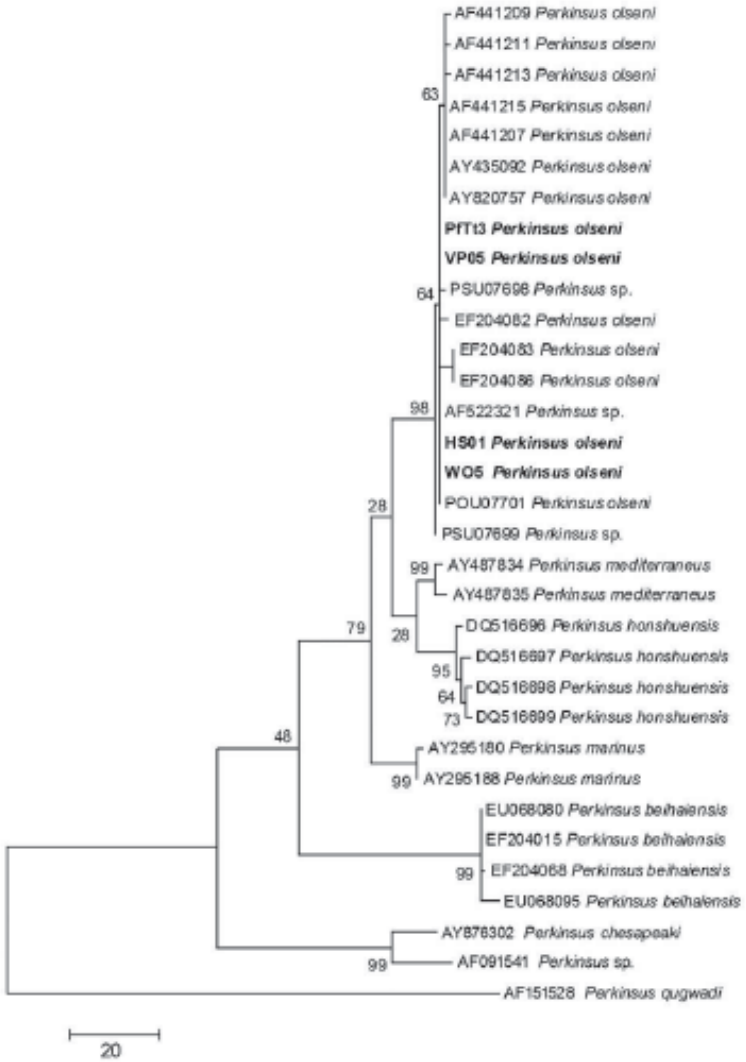
The pairwise genetic distance between the present 4 isolates of *P. olseni* from *P. fucata* appeared to be 0, indicating that they belong to the same species. The pairwise genetic distance between the present *P. olseni* isolates and other members of *P. olseni* group (from different hosts) studied, ranged from 0 to 0.009 (mean, 0.0027), indicating its affiliation to the *P. olseni* clade. On the other hand, the significant variations observed in the pairwise genetic distance between the present isolate and the **other species of *Perkinsus***, clearly showed that they were all taxonomically distinct.

Mean pairwise genetic distances between the various species of *Perkinsus*.

| | *P. ols* | *P. med* | *P. hon* | *P. mar* | *P. bei* | *P. Che* | *P. qug* |
|---|---|---|---|---|---|---|---|
| Present isolate | 0.0027 | 0.0365 | 0.0485 | 0.0480 | 0.1343 | 0.1530 | 0.4280 |
| *P. olseni* | | 0.0382 | 0.0502 | 0.0498 | 0.1360 | 0.1541 | 0.4275 |
| *P. mediterraneus* | | | 0.0320 | 0.0470 | 0.1230 | 0.1435 | 0.4430 |
| *P. honshuensis* | | | | 0.0450 | 0.1173 | 0.1395 | 0.4388 |
| *P. marinus* | | | | | 0.1328 | 0.1475 | 0.4255 |
| *P. beihaiensis* | | | | | | 0.1825 | 0.4858 |
| *P. chesapeaki* | | | | | | | 0.4905 |
| *P. qugwadi* | | | | | | | |

Further, maximum parsimony and neighbor joining analysis of the nucleotide sequences of the ITS region of the present parasite showed it to be *Perkinsus olseni* and sequences from all the 4 samples were identical and were positioned along with the members of the *P. olseni* clade. The topologies of the trees generated with the distance and parsimony analyses were similar. The study has proved that the protozoan parasite infecting the pearl oyster was *Perkinsus olseni* beyond any doubt. This forms the first report on the existence of *P. olseni*, an OIE listed protozoan parasite in *P. fucata* from the southeast coast of India.

This study clearly proved that molecular diagnostic techniques can be extremely useful in the diagnosis of protozoan parasites which are otherwise difficult to diagnose using conventional techniques. Further the specific taxonomic status of the pathogen can also be revealed without any scope for ambiguity. Techniques like nested PCR can enhance the sensitivity of molecular diagnostic techniques greatly.




*Phylogenetic tree constructed using maximum parsimony method*

## Case study 2 :

## Diagnosis of WSSV in shrimps

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# 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; *nv*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 *nv*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 $mm^3$) 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) | *P. monodon* | Sub-adult | Cultured | 'Virulent' (V1) |
| Marakkanam (TN) | *P. monodon* | Sub-adult | Cultured | 'Virulent' (V2) |
| Mahabalipuram (TN) | *P. monodon* | Adult | Cultured | 'Non-virulent' (NV1) |
| Kovalam (TN) | *P. monodon* | Adult | Cultured | 'Non-virulent' (NV2) |
| Kattur (TN) | *P. monodon* | Adult | Cultured | 'Virulent' (V3) |
| Gudur (AP) | *P. monodon* | Sub-adult | Cultured | 'Virulent' (V4) |
| Nellore (AP) | *P. monodon* | Adult | Cultured | 'Virulent' (V5) |
| Kakinada (AP) | *P. monodon* | Adult | Cultured | 'Virulent' (V6) |
| Bhimavaram (AP) | *P. monodon* | 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-μm-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 × *g* for 5 min at 4 °C. The supernatant was centrifuged at 100 000 × *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 overlayered on the top of a 10–50% (w/v) continuous sucrose gradient and centrifuged at 123 000 × *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 × *g* for 1 h. The pellet was then resuspended in 100 μL 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 μm 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 μg 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 *nv*WSSV) under sterile conditions. The haemolymph was allowed to clot, and the serum was separated by centrifugation at 3000 × *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, Vlak & van Hulten 2004; Witteveldt, Vlak & van Hulten 2004) and dsRNA and siRNA targeting this gene (Robalino, Bartlett, Shepard, Prior, Jaramillo, Scura, Chapman, Gross, Browdy & Warr 2005; Westenberg, Heinhuis, Zuidema & Vlak 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′-AGA*GA ATTC*ATGGATCTTTCTTTCAC-3′ (*Eco*RI site in italics) and reverse primer 5′-CAC*GTCGAC*TTACTCGGTCTC 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 27–29 °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 *nv*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 × *g* for 10 min at 4 °C. The supernatant was filtered through a 0.45 µm 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 ± 0.5 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 animals $tank^{-1}$). Each shrimp from the test group was injected with 50 µL 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 *nv*WSSV showed typical clinical signs of WSSV infection. Virulent WSSV-infected shrimps showed white spots on the carapace, reddish discolouration 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 *nv*WSSV. Furthermore, these animals were found to be healthy with normal feed intake and survived until harvest.

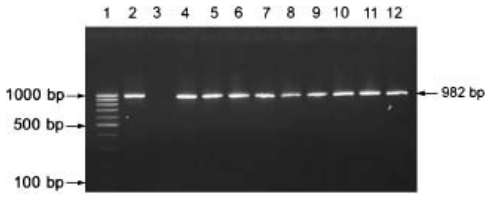


**Figure 1** Detection of WSSV in the shrimp samples collected at different places. All samples were positive by first-step PCR. Lane1: 100 bp DNA ladder, lane 2: positive control, lane 3: negative control, lanes 4–12: shrimp tissue samples in order of collection site (please see Table 1 for order).

### 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 *nv*WSSV-infected (Fig. 2b) shrimps were similar. These changes were characterized by nuclear hypertrophy, chromatin margination, variable multifocal necrosis and haemocyte encapsulations. In *nv*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 *nv*WSSV-infected (Fig. 3b) tissues showed similarity in size and morphology. The size of the larger virion ranged between 112 and 268 ± 34 nm in length, whereas the smaller virion ranged between 98 and 260 ± 30 nm in length. The size of the nucleocapsid varied from 82 to 246 ± 35 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

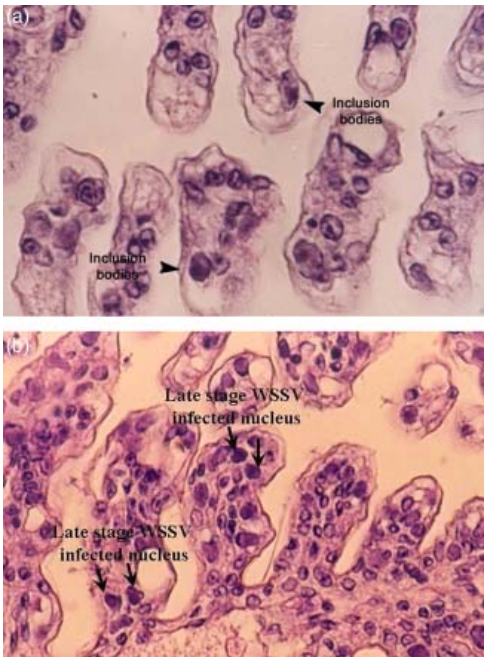


**Figure 2** (a) Light photomicrograph of histological section through a region of non-virulent WSSV-infected gills of *Penaeus monodon*. Cells showing eosinophilic intracellular inclusions (arrow heads) surrounded by marginated basophilic chromatin are observed (b) Histological section through a region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

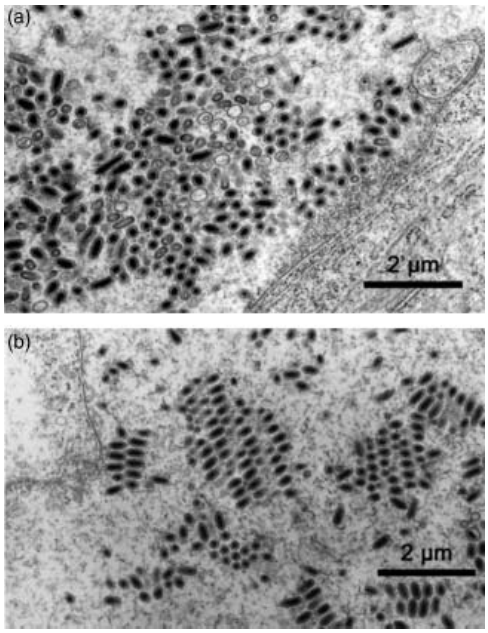


**Figure 3** Transmission electron micrograph of ultrathin section of WSSV-infected stomach epithelial cells of *Penaeus monodon*. (a) *v*WSSV-infected shrimp tissue section. (b) *nv*WSSV-infected shrimp tissue section. Note the arrangement of viral particles in paracrystalline array.

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 *v*WSSV and *nv*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 Hulten, Westenberg, Goodall & Vlak 2000). The protein profiles obtained from the *v*WSSV and *nv*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 *nv*WSSV-infected shrimp (Fig. 6), which were not present in the haemolymph protein of shrimp affected with *v*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 *nv*WSSV shrimp protein.

### Sequencing and comparison of VP28

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

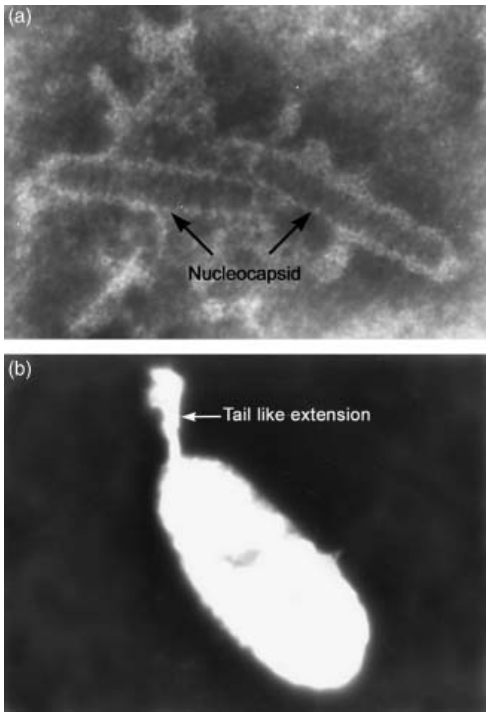


**Figure 4** Electron micrograph of negatively stained WSSV purified by sucrose gradient. (a) Enveloped rod-shaped nucleocapsid is clearly observed. There is a distinct vertical helix located horizontally along the axis of the nucleocapisd core. (b) Complete virion with the tail-like extension is observed.

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 *nv*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 *nv*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 *nv*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 *nv*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 *nv*WSSV virions showed similar morphologies, with no discernible difference in size or shape.

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

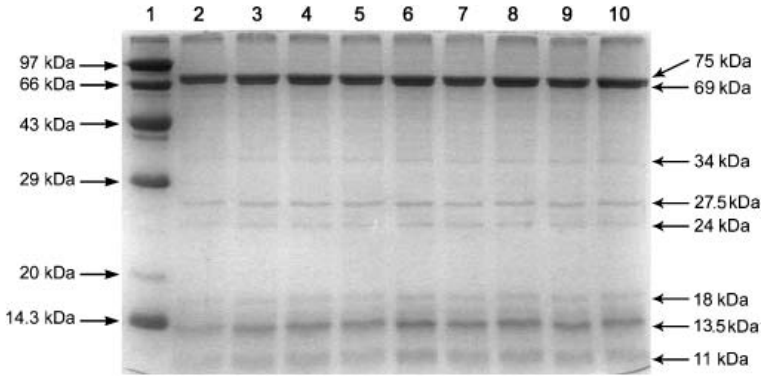


**Figure 5** SDS-PAGE protein profile of the nine WSSV isolates. Lane 1: protein molecular weight marker; Lanes 2, 3, 6, 7, 8 and 9: V1, V2, V3, V4, V5 and V6 respectively; Lanes 4, 5 and 10: NV1, NV2 and NV3, respectively, where V, virulent isolate and NV, non-virulent isolate (see Table 1 for details). 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.

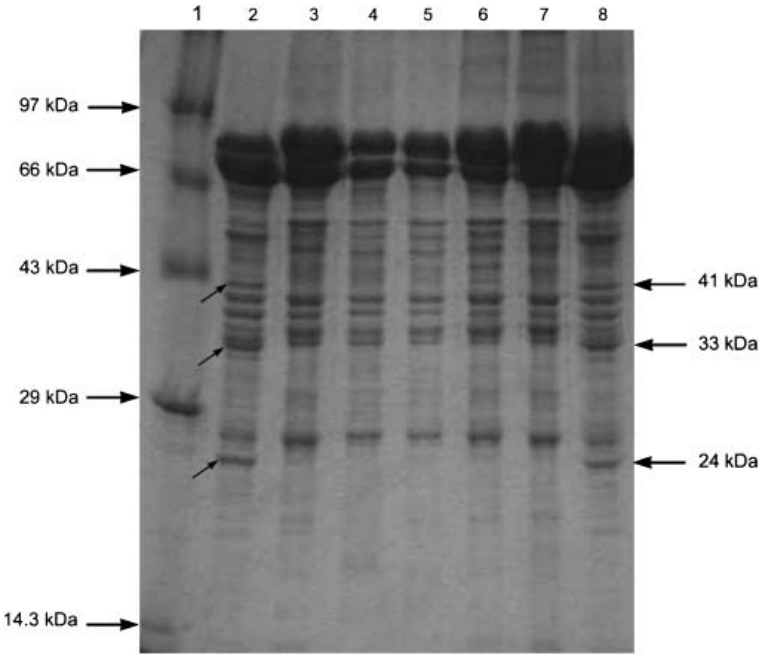


**Figure 6** SDS-PAGE profile of shrimp haemolymph from *v*WSSV- and *nv*WSSV-infected animals. Lane 1: medium range protein molecular weight marker, Lanes 2 and 8: haemolymph of shrimp infected by *nv*WSSV, Lanes 3–7: haemolymph of shrimps infected by *v*WSSV. The three additional protein bands obtained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

jor 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 *vp*28 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 *nv*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 *Pm*AV 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 '*nv*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 *nv*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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